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(54) Title: ANTIBODY-TOXIN CONJUGATES

(57) Abstract: The present invention provides conjugates formed between toxins and sugars and toxins and peptide, such as antibodies. In an exemplary embodiment, a toxin-sugar construct is conjugated to an antibody through and intact glycosyl linking group.

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ANTIBODY-TOXIN CONJUGATES

CROSS-REFERENCES TO RELATED APPLICATIONS

This is a no-Provisional filing of U.S. Patent application N. 60/490,168 filed July 25, 2003, and U.S. Patent Application No. 60/499,448 filed September 2, 2003, each of which is incorporated by reference herein in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0001] NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.

[0002] NOT APPLICABLE

BACKGROUND OF THE INVENTION

The administration of glycosylated and non-glycosylated peptides for engendering a particular physiological response is well known in the medicinal arts. Among the best known peptides utilized for this purpose is insulin, which is used to treat diabetes. Enzymes have also been used for their therapeutic benefits. A principal factor, which has limited the use of therapeutic peptides is the immunogenic nature of most peptides. In a patient, an immunogenic response to an administered peptide can neutralize the peptide and/or lead to the development of an allergic response in the patient. Other deficiencies of therapeutic glycopeptides include suboptimal potency and rapid clearance rates. The problems inherent in peptide therapeutics are recognized in the art, and various methods of eliminating the problems have been investigated. To provide soluble peptide therapeutics, synthetic polymers have been attached to the peptide backbone.

Poly(ethylene glycol) ("PEG") is an exemplary polymer that has been conjugated to peptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides. For example, U.S. Pat. No. 4,179,337 (Davis *et al.*) concerns non-immunogenic polypeptides, such as enzymes and

peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole polypeptide and at least 15% of the physiological activity is maintained. In addition, the clearance time in circulation is prolonged due to the increased size of the PEG-conjugate of the polypeptides in question.

WO 93/15189 (Veronese *et al.*) concerns a method to maintain the activity of polyethylene glycol-modified proteolytic enzymes by linking the proteolytic enzyme to a macromolecularized inhibitor. The conjugates are intended for medical applications.

The principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. For example, U.S. Patent No. 4,088,538 discloses an enzymatically active polymer-enzyme conjugate of an enzyme covalently bound to PEG. Similarly, U.S. Patent No. 4,496,689 discloses a covalently attached complex of α-1 proteinase inhibitor with a polymer such as PEG or methoxypoly(ethyleneglycol) ("MPEG"). Abuchowski *et al.* (*J. Biol. Chem.* **252**: 3578 (1977) discloses the covalent attachment of MPEG to an amine group of bovine serum albumin. U.S. Patent No. 4,414,147 discloses a method of rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride). PCT WO 87/00056 discloses conjugation of PEG and poly(oxyethylated) polyols to such proteins as interferon-β, interleukin-2 and immunotoxins. EP 154,316 discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of the lymphokine. U.S. Patent No. 4,055,635 discloses pharmaceutical compositions of a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as a polysaccharide.

Another mode of attaching PEG to peptides is through the non-specific oxidation of glycosyl residues on a glycopeptide. The oxidized sugar is utilized as a locus for attaching a PEG moiety to the peptide. For example M'Timkulu (WO 94/05332) discloses the use of an amino-PEG to add PEG to a glycoprotein. The glycosyl moieties are randomly oxidized to the corresponding aldehydes, which are subsequently coupled to the amino-PEG.

In each of the methods described above, poly(ethyleneglycol) is added in a random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivitization strategy that results in the formation of a specifically labeled, readily characterizable, essentially homogeneous product. A promising route to preparing specifically labeled peptides is through the use of enzymes, such as glycosyltransferases to append a modified sugar moiety onto a peptide.

Enzyme-based syntheses have the advantages of regioselectivity and stereoselectivity. Moreover, enzymatic syntheses are performed using unprotected substrates. Two principal classes of enzymes are used in the synthesis of carbohydrates, glycosyltransferases (*e.g.*, sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as exoglycosidases (*e.g.*, β-mannosidase, β-glucosidase), and endoglycosidases (*e.g.*, Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to prepare carbohydrates. For a general review, *see,* Crout *et al., Curr. Opin. Chem. Biol.* **2**: 98-111 (1998).

Glycosyltransferases modify the oligosaccharide structures on glycopeptides. Glycosyltransferases are effective for producing specific products with good stereochemical and regiochemical control. Glycosyltransferases have been used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on glycopeptides produced in mammalian cells. For example, the terminal oligosaccharides of glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example, β-1,4-galactosyltransferase was used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of carbohydrates (*see, e.g.*, Wong *et al., J. Org. Chem.* **47**: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of α-sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneuraminic acid to the 3-OH or 6-OH of galactose (*see, e.g.*, Kevin *et al., Chem. Eur. J.* **2**: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific hydroxyl of a saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase (Ichikawa *et al., J. Am. Chem. Soc.* **114**: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use *see,* Koeller *et al., Nature Biotechnology* **18**: 835-841 (2000). *See* also, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

Glycosidases can also be used to prepare saccharides. Glycosidases normally catalyze the hydrolysis of a glycosidic bond. Under appropriate conditions, however, they can be used to form this linkage. Most glycosidases used for carbohydrate synthesis are exoglycosidases; the glycosyl transfer occurs at the non-reducing terminus of the substrate.

The glycosidase takes up a glycosyl donor in a glycosyl-enzyme intermediate that is either intercepted by water to give the hydrolysis product, or by an acceptor, to give a new glycoside or oligosaccharide. An exemplary pathway using an exoglycosidase is the synthesis of the core trisaccharide of all N-linked glycopeptides, including the notoriously difficult β-mannoside linkage, which was formed by the action of β-mannosidase (Singh *et al., Chem. Commun.* 993-994 (1996)).

In another exemplary application of the use of a glycosidase to form a glycosidic linkage, a mutant glycosidase has been prepared in which the normal nucleophilic amino acid within the active site is changed to a non-nucleophilic amino acid. The mutant enzymes do not hydrolyze glycosidic linkages, but can still form them. The mutant glycosidases are used to prepare oligosaccharides using an α-glycosyl fluoride donor and a glycoside acceptor molecule (Withers et al., U.S. Patent No. 5,716,812). Although the mutant glycosidases are useful for forming free oligosaccharides, it has yet to be demonstrated that such enzymes are capable of appending glycosyl donors onto glycosylated or non-glycosylated peptides, nor have these enzymes been used with unactivated glycosyl donors.

Although their use is less common than that of the exoglycosidases, endoglycosidases are also utilized to prepare carbohydrates. Methods based on the use of endoglycosidases have the advantage that an oligosaccharide, rather than a monosaccharide, is transferred. Oligosaccharide fragments have been added to substrates using *endo*-β-N-acetylglucosamines such as *endo*-F, *endo*-M (Wang *et al., Tetrahedron Lett.* **37**: 1975-1978); and Haneda *et al., Carbohydr. Res.* **292**: 61-70 (1996)).

In addition to their use in preparing carbohydrates, the enzymes discussed above are applied to the synthesis of glycopeptides as well. The synthesis of a homogenous glycoform of ribonuclease B has been published (Witte K. *et al., J. Am. Chem. Soc.* **119**: 2114-2118 (1997)). The high mannose core of ribonuclease B was cleaved by treating the glycopeptide with endoglycosidase H. The cleavage occurred specifically between the two core GlcNAc residues. The tetrasaccharide sialyl Lewis X was then enzymatically rebuilt on the remaining GlcNAc anchor site on the now homogenous protein by the sequential use of β-1,4-galactosyltransferase, α-2,3-sialyltransferase and α-1,3-fucosyltransferase V. Each enzymatically catalyzed step proceeded in excellent yield.

Methods combining both chemical and enzymatic synthetic elements are also known. For example, Yamamoto and coworkers (*Carbohydr. Res.* **305**: 415-422 (1998))

reported the chemoenzymatic synthesis of the glycopeptide, glycosylated Peptide T, using an endoglyosidase. The N-acetylglucosaminyl peptide was synthesized by purely chemical means. The peptide was subsequently enzymatically elaborated with the oligosaccharide of human transferrin glycopeptide. The saccharide portion was added to the peptide by treating it with an endo-β-N-acetylglucosaminidase. The resulting glycosylated peptide was highly stable and resistant to proteolysis when compared to the peptide T and N-acetylglucosaminyl peptide T.

The use of glycosyltransferases to modify peptide structure with reporter groups has been explored. For example, Brossmer *et al.* (U.S. Patent No. 5,405,753) discloses the formation of a fluorescent-labeled cytidine monophosphate ("CMP") derivative of sialic acid and the use of the fluorescent glycoside in an assay for sialyl transferase activity and for the fluorescent-labeling of cell surfaces, glycoproteins and gangliosides. Gross *et al.* (*Analyt. Biochem.* **186**: 127 (1990)) describe a similar assay. Bean *et al.* (U.S. Patent No. 5,432,059) discloses an assay for glycosylation deficiency disorders utilizing reglycosylation of a deficiently glycosylated protein. The deficient protein is reglycosylated with a fluorescent-labeled CMP glycoside. Each of the fluorescent sialic acid derivatives is substituted with the fluorescent moiety at either the 9-position or at the amine that is normally acetylated in sialic acid. The methods using the fluorescent sialic acid derivatives are assays for the presence of glycosyltransferases or for non-glycosylated or improperly glycosylated glycoproteins. The assays are conducted on small amounts of enzyme or glycoprotein in a sample of biological origin. The enzymatic derivatization of a glycosylated or non-glycosylated peptide on a preparative or industrial scale using a modified sialic acid has not been disclosed or suggested.

Considerable effort has also been directed towards the modification of cell surfaces by altering glycosyl residues presented by those surfaces. For example, Fukuda and coworkers have developed a method for attaching glycosides of defined structure onto cell surfaces. The method exploits the relaxed substrate specificity of a fucosyltransferase that can transfer fucose and fucose analogs bearing diverse glycosyl substrates (Tsuboi *et al.*; *J. Biol. Chem.* **271**: 27213 (1996)).

Enzymatic methods have also been used to activate glycosyl residues on a glycopeptide towards subsequent chemical elaboration. The glycosyl residues are typically activated using galactose oxidase, which converts a terminal galactose residue to the corresponding aldehyde. The aldehyde is subsequently coupled to an amine-containing

modifying group. For example, Casares *et al.* (*Nature Biotech.* **19**: 142 (2001)) have attached doxorubicin to the oxidized galactose residues of a recombinant MHCII-peptide chimera.

Glycosyl residues have also been modified to bear ketone groups. For example, Mahal and co-workers (*Science* **276**: 1125 (1997)) have prepared N-levulinoyl mannosamine ("ManLev"), which has a ketone functionality at the position normally occupied by the acetyl group in the natural substrate. Cells were treated with the ManLev, thereby incorporating a ketone group onto the cell surface. *See,* also Saxon *et al., Science* **287**: 2007 (2000); Hang *et al., J. Am. Chem. Soc.* **123**: 1242 (2001); Yarema *et al., J. Biol. Chem.* **273**: 31168 (1998); and Charter *et al., Glycobiology* **10**: 1049 (2000).

The methods of modifying cell surfaces have not been applied in the absence of a cell to modify a glycosylated or non-glycosylated peptide. Moreover, the methods of cell surface modification are not utilized for the enzymatic incorporation preformed modified glycosyl donor moiety into a peptide. Moreover, none of the cell surface modification methods are practical for producing glycosyl-modified peptides on an industrial scale.

Despite the efforts directed toward the enzymatic elaboration of saccharide structures, there remains still a need for an industrially practical method for the modification of glycosylated and non-glycosylated peptides with modifying groups such as toxins. Of particular interest are methods in which the modified peptide has improved properties, which enhance its use as a therapeutic or diagnostic agent. The present invention fulfills these and other needs.

BRIEF SUMMARY OF THE INVENTION

In response to the need for improved site-specific delivery of toxins to the loci of disease, the present invention provides antibodies that are modified with toxins. The invention provides a unique class of conjugates in which the toxin is attached to the antibody through a glycosyl linking group, e.g., an intact glycosyl linking group, which is attached to the peptide (or to an acceptor moiety attached to the peptide, e.g. a spacer or amplifier) utilizing an enzymatically-mediated coupling reaction.

Site-specific and target-oriented delivery of therapeutic agents is desirable for the purpose of treating a wide variety of human diseases, such as different types of malignancies and certain neurological disorders. Such procedures are accompanied by fewer side effects and a higher efficacy of drug. Various principles have been relied on in

designing these delivery systems. For a review, *see* Garnett, *Advanced Drug Delivery Reviews* **53**:171-216 (2001).

Tumor surface antigens make possible the development of therapeutic approaches in which tumor cells displaying definable surface antigens are specifically targeted and killed.

Thus, in a first aspect, the present invention provides a peptide conjugate which the sugar-toxin construct (modified sugar) is attached to a peptide. For example, the invention provides a peptide conjugate having the formula:

Ab—G—L—T

wherein Ab is an antibody, or other targeting moiety; G is a glycosyl linking group, e.g., an intact glycosyl linking group, covalently joining Ab to L; L is a bond or a spacer moiety covalently joining G to T; and T is a toxin, or other therapeutic agent.

The spacer moiety can be any of a wide variety of molecular structures, and will be at least bifunctional to permit attachment to both G and T, optionally through linking groups. Preferred spacers are those which are cleaved *in vivo* by the biological environment within a relatively short period of time following delivery of the conjugate to its target. Spacers with multiple binding functionalities at the toxin end accommodate a multitude of toxins. Spacers of this type serve an amplifying function. Exemplary spacers include those which are non-toxic and non-immunogenic, while those which are particularly useful will be those which have further properties which impart beneficial properties to the conjugate. In addition to the property of *in vivo* cleavability mentioned above, a property of interest is the hydrophilicity of the conjugate. Still other useful properties are low antigenicity and increased molecular weight. Spacers with still further properties can be utilized to advantage as well, as will be readily apparent to those skilled in the art.

In a second aspect, the invention provides a compound having the formula:

S—L—T

wherein S is a nucleotide sugar; L is a bond or a spacer moiety covalently joining S to T; and T is a toxin moiety.

Other objects and advantages of the invention will be apparent to those of skill in the art from the detailed description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a scheme showing an exemplary embodiment of the invention in which a carbohydrate residue on a glycoprotein is "trimmed back" and a modified sugar bearing a water soluble polymer is conjugated to one or more of the sugar residues exposed by the "trimming back." In the scheme, high mannose is trimmed back to the first generation biantennary structure.

FIG. 2 is a scheme similar to that shown in **FIG. 1**, in which the high mannose structure is "trimmed back" to the mannose from which the biantennary structure branches.

FIG. 3 is a scheme similar to that shown in **FIG. 1**, in which high mannose is "trimmed back" to the GlcNAc to which the first mannose is attached.

FIG. 4 is a scheme similar to that shown in **FIG. 1**, in which high mannose is trimmed back to the first GlcNAc attached to the Asn of the peptide.

FIG. 5 is a scheme in which a N-linked carbohydrate is trimmed back and subsequently derivatized with a modified sugar moiety (GlcNAc) bearing a water-soluble polymer.

FIG. 6 is a scheme in which a N-linked carbohydrate is trimmed back and subsequently derivatized with a sialic acid moiety bearing a water-soluble polymer.

FIG. 7 is a scheme in which a N-linked carbohydrate is trimmed back and subsequently derivatized with one or more sialic acid moieties, and terminated with a sialic acid derivatized with a water-soluble polymer.

FIG. 8 is a scheme in which an O-linked saccharide is "trimmed back" and subsequently conjugated to a modified sugar bearing a water soluble polymer. In the exemplary scheme, the carbohydrate moiety is "trimmed back" to the first generation of the biantennary structure.

FIG. 9 is an exemplary scheme for trimming back the carbohydrate moiety of an O-linked glycopeptide to produce a mannose available for conjugation with a modified sugar having a water-soluble polymer attached thereto.

FIG. 10 shows a sampling of the array of reactions available for derivatizing a saccharide with a modified sugar.

FIG. 11 is a table that includes exemplary toxins of use in the conjugates of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

PEG, poly(ethyleneglycol); PPG, poly(propyleneglycol); Ara, arabinosyl; Fru, fructosyl; Fuc, fucosyl; Gal, galactosyl; GalNAc, N-acetylgalactosaminyl; Glc, glucosyl; GlcNAc, N-acetylglucosaminyl; Man, mannosyl; ManAc, mannosaminyl acetate; Xyl, xylosyl; and NeuAc, sialyl (N-acetylneuraminyl).

Definitions

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references (*see generally*, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

All oligosaccharides described herein are described with the name or abbreviation for the non-reducing saccharide (*i.e.*, Gal), followed by the configuration of the glycosidic bond (α or β), the ring bond (1 or 2), the ring position of the reducing saccharide involved in the bond (2, 3, 4, 6 or 8), and then the name or abbreviation of the reducing saccharide (*i.e.*, GlcNAc). Each saccharide is preferably a pyranose. For a review of standard glycobiology nomenclature *see, Essentials of Glycobiology* Varki *et al.* eds. CSHL Press (1999).

Oligosaccharides are considered to have a reducing end and a non-reducing end, whether or not the saccharide at the reducing end is in fact a reducing sugar. In accordance with accepted nomenclature, oligosaccharides are depicted herein with the non-reducing end on the left and the reducing end on the right.

The term "sialic acid" refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.* (1986) *J. Biol. Chem.* **261**: 11550-11557; Kanamori *et al., J. Biol. Chem.* **265**: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C_1-C_6 acyl-Neu5Ac like 9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, *see, e.g.*, Varki, *Glycobiology* **2**: 25-40 (1992); *Sialic Acids: Chemistry, Metabolism and Function*, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published October 1, 1992.

"Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. Additionally, unnatural amino acids, for example, β-alanine, phenylglycine and homoarginine are also included. Amino acids that are not gene-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the invention. All of the amino acids used in the present invention may be either the D - or L - isomer. The L -isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and unglycosylated peptides. Also included are petides that are incompletely glycosylated by a system that expresses the peptide. For a general review, *see,* Spatola, A. F., in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

The term "peptide conjugate," refers to species of the invention in which a peptide is conjugated with a modified sugar as set forth herein.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have

the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

As used herein, the term "modified sugar," refers to a naturally- or non-naturally-occurring carbohydrate that is enzymatically added onto an amino acid or a glycosyl residue of a peptide in a process of the invention. The modified sugar is selected from a number of enzyme substrates including, but not limited to sugar nucleotides (mono-, di-, and tri-phosphates), activated sugars (*e.g.*, glycosyl halides, glycosyl mesylates) and sugars that are neither activated nor nucleotides. The "modified sugar" is covalently functionalized with a "modifying group." Useful modifying groups include, but are not limited to, water-soluble polymers, therapeutic moieties, e.g., toxins, diagnostic moieties, biomolecules and the like. The modifying group is preferably not a naturally occurring, or an unmodified carbohydrate. The locus of functionalization with the modifying group is selected such that it does not prevent the "modified sugar" from being added enzymatically to a peptide.

The term "water-soluble" refers to moieties that have some detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences of be composed of a single amino acid, e.g. poly(lysine). Similarly, saccharides can be of mixed sequence or composed of a single saccharide subunit, e.g, dextran, amylose, chitosan, and poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol). Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic) acid is a representative poly(carboxylic acid)

The term, "glycosyl linking group," as used herein refers to a glycosyl residue to which an acyl-containing modifying group (e.g., PEG moiety, therapeutic moiety, biomolecule) is covalently attached; the glycosyl linking group joins the modifying group to the remainder of the conjugate. In the methods of the invention, the "glycosyl linking group" is formed by the covalent modification, via an enzymatic acylation reaction of a glycosyl residue, thereby linking the agent to an amino acid and/or glycosyl residue on the peptide. The glycosyl linking group can be a saccharide-derived structure that is degraded or degraded

and modified prior to the addition of the modifying group (e.g., oxidation→Schiff base formation→reduction). Alternatively, the glycosyl linking group may be intact. An "intact glycosyl linking group" refers to a linking group that is derived from a glycosyl moiety in which the saccharide monomer that links the modifying group and to the remainder of the conjugate is not degraded, e.g., oxidized, e.g., by sodium metaperiodate to create a locus of attachment for the modifying group. "Intact glycosyl linking groups" of the invention may be derived from a naturally occurring oligosaccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure.

Exemplary methods of forming "glycosyl linking groups" are disclosed in commonly owned copending Patent Application No. PCT/US02/32263, filed October 9, 2002; Provisional Patent Applications 60/448,381, filed February 19, 2003 (converted to non-provisional application, same filing date, serial number not yet assigned); 60/438,582, filed January 6, 2003 (converted to non-provisional application, same filing date, serial number not yet assigned); 60/407,527, filed August 28, 2002; 60/404,249, filed August 16, 2002; 60/396,594, filed July 17, 2002; 60/391,777, filed June 25, 2002; 60/387,292, filed June 7, 2002; 60/334,301, filed November 28, 2001; 60/334,233, filed November 28, 2001; 60/344,692, filed October 19, 2001; 60/328,523, filed October 10, 2001; and U.S. Patent Application No. 10/410913, filed April 9, 2003.

The term "targeting moiety," as used herein, refers to species that will selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or conjugate, ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an agent to a particular tissue or region are known to those of skill in the art. Exemplary targeting moieties include antibodies, antibody fragments, transferrin, HS-glycoprotein, coagulation factors, serum proteins, β-glycoprotein, G-CSF, GM-CSF, M-CSF, EPO and the like.

As used herein, "therapeutic moiety" means any agent useful for therapy including, but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is bound to a carrier, e.g, multivalent agents. Therapeutic moiety also includes proteins and constructs that include proteins. Exemplary proteins include, but are not limited to, Erythropoietin (EPO), Granulocyte Colony Stimulating Factor (GCSF), Granulocyte Macrophage Colony

Stimulating Factor (GMCSF), Interferon (e.g., Interferon-α, -β, -γ), Interleukin (e.g., Interleukin II), serum proteins (e.g., Factors VII, VIIa, VIII, IX, and X), Human Chorionic Gonadotropin (HCG), Follicle Stimulating Hormone (FSH) and Lutenizing Hormone (LH) and antibody fusion proteins (e.g. Tumor Necrosis Factor Receptor ((TNFR)/Fc domain fusion protein)).

As used herein, "anti-tumor drug" means any agent useful to combat cancer including, but not limited to, cytotoxins and agents such as antimetabolites, alkylating agents, anthracyclines, antibiotics, antimitotic agents, procarbazine, hydroxyurea, asparaginase, corticosteroids, interferons and radioactive agents. Also encompassed within the scope of the term "anti-tumor drug," are conjugates of peptides with anti-tumor activity, *e.g.* TNF-α. Conjugates include, but are not limited to those formed between a therapeutic protein and a glycoprotein of the invention. A representative conjugate is that formed between PSGL-1 and TNF-α.

As used herein, "toxin", "cytotoxin" or "cytotoxic agent" means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Other toxins include, for example, ricin, CC-1065 and analogues, the duocarmycins. Still other toxins include diptheria toxin, and snake venom (e.g., cobra venom).

As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (e.g. EDTA, DTPA, DOTA, NTA, HDTA, *etc.* and their phosphonate analogs such as DTPP, EDTP, HDTP, NTP, *etc*). *See*, for example, Pitt *et al.*, "The Design of Chelating Agents for the Treatment of Iron Overload," In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF

MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge,1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein.

Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. *See*, for example, Meares *et al.*, "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS;" Feeney, *et al.*, Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina *et al., Bioconjugate Chem.*, **9:** 108-117 (1998); Song *et al., Bioconjugate Chem.*, **8:** 249-255 (1997).

As used herein, "pharmaceutically acceptable carrier" includes any material, which when combined with the conjugate retains the conjugates' activity and is non-reactive with the subject's immune systems. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intralesional, intranasal or subcutaneous administration, or the implantation of a slow-release device *e.g.*, a mini-osmotic pump, to the subject. Adminsitration is by any route including parenteral, and transmucosal (e.g., oral, nasal, vaginal, rectal, or transdermal). Parenteral administration includes, e.g., intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Moreover, where injection is to treat a tumor, e.g., induce apoptosis, administration may be directly to the tumor and/or into tissues surrounding the tumor. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

The term "isolated" refers to a material that is substantially or essentially free from components, which are used to produce the material. For peptide conjugates of the invention, the term "isolated" refers to material that is substantially or essentially free from

components, which normally accompany the material in the mixture used to prepare the peptide conjugate. "Isolated" and "pure" are used interchangeably. Typically, isolated peptide conjugates of the invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

When the peptide conjugates are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% purity.

Purity is determined by any art-recognized method of analysis (*e.g.*, band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, or a similar means).

"Essentially each member of the population," as used herein, describes a characteristic of a population of peptide conjugates of the invention in which a selected percentage of the modified sugars added to a peptide are added to multiple, identical acceptor sites on the peptide. "Essentially each member of the population" speaks to the "homogeneity" of the sites on the peptide conjugated to a modified sugar and refers to conjugates of the invention, which are at least about 80%, preferably at least about 90% and more preferably at least about 95% homogenous.

"Homogeneity," refers to the structural consistency across a population of acceptor moieties to which the modified sugars are conjugated. Thus, in a peptide conjugate of the invention in which each modified sugar moiety is conjugated to an acceptor site having the same structure as the acceptor site to which every other modified sugar is conjugated, the peptide conjugate is said to be about 100% homogeneous. Homogeneity is typically expressed as a range. The lower end of the range of homogeneity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

When the peptide conjugates are more than or equal to about 90% homogeneous, their homogeneity is also preferably expressed as a range. The lower end of the range of homogeneity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% homogeneity. The purity of the peptide conjugates is typically determined by one or more methods known to those of skill in the art, e.g., liquid chromatography-mass

spectrometry (LC-MS), matrix assisted laser desorption mass time of flight spectrometry (MALDITOF), capillary electrophoresis, and the like.

"Substantially uniform glycoform," "substantially homogeneous derivatization pattern," or a "substantially uniform glycosylation pattern," when referring to a glycopeptide species, refers to the percentage of acceptor moieties that are glycosylated by the glycosyltransferase of interest (*e.g.*, fucosyltransferase). For example, in the case of a α1,2 fucosyltransferase, a substantially uniform fucosylation pattern exists if substantially all (as defined below) of the Galβ1,4-GlcNAc-R and sialylated analogues thereof are fucosylated in a peptide conjugate of the invention. It will be understood by one of skill in the art, that the starting material may contain glycosylated acceptor moieties (*e.g.*, fucosylated Galβ1,4-GlcNAc-R moieties). Thus, the calculated percent glycosylation will include acceptor moieties that are glycosylated by the methods of the invention, as well as those acceptor moieties already glycosylated in the starting material.

The term "substantially" in the above definitions of "substantially uniform" generally means at least about 40%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

The Embodiments

This invention provides a novel class of tissue targeted peptide-therapeutic agent conjugates. The conjugates are formed between toxins and sugars, or sugar nucleotides or between these species and a peptide. The conjugates can include a single toxin moiety or they can be constructs which include a plurality of toxins joined to a macromolecular or polymeric amplifying moiety, optionally, through one or more spacer groups.

The present invention provides peptide-toxin conjugates in which the toxin (or a spacer attached to the toxin) is linked to the peptide via a glycosyl linking group, preferably an intact glycosyl linking group. The present method is in clear contrast to prior methods which rely upon the oxidative degradation of a saccharyl moiety, followed by Schiff base formation and reduction to form conjugates between selected agents and the glycosyl groups of peptides.

As set forth in the figures appended hereto, the conjugates of the invention can include intact glycosyl linking groups that are mono- or multi-valent (e.g., antennary structures). Thus, conjugates of the invention include species in which a selected moiety is

attached to a peptide via a monovalent glycosyl linking group. Also included within the invention are conjugates in which more than one selected moiety (*e.g.*, toxin, toxin-linker) is attached to a peptide via a multivalent linking group.

Thus, in a first aspect, the invention provides a compound having the formula:

Ab—G—L—T

wherein Ab is an antibody; G is an intact glycosyl linking group covalently joining Ab to L; L is a bond or a spacer moiety covalently joining G to T; and T is a toxin.

The spacer group can be any of a wide variety of molecular structures, and will be at least bifunctional to permit attachment to both G and T, optionally through linking groups. Preferred spacers are those which are cleaved *in vivo* naturally occurring biological processes within a relatively short period of time after the conjugate is delivered to its target. Spacers with multiple binding functionalities at the toxin end accommodate a multitude of toxins. Spacers of this type serve an amplifying function. Exemplary spacers include those which are non-toxic and non-immunogenic, while those which are particularly useful will be those which have further properties which impart beneficial properties to the conjugate. In addition to the property of *in vivo* cleavability mentioned above, a property of interest is the hydrophilicity of the conjugate. Thus, in certain embodiments, the spacer includes a hydrophilic component. Still other useful properties are low antigenicity and increased molecular weight. Spacers with still further properties can be utilized to advantage as well.

The spacer, L, may be either a straight-chain or a branched-chain structure. Preferred L groups are those which include a straight chain within their structures, either as the entire spacer group or as the backbone of a branched-chain group. The straight chain may be a chain of carbon atoms or of carbon atoms interrupted with one or more hetero atoms such as oxygen atoms, sulfur atoms or nitrogen atoms. The bonds forming the chain may be single bonds or double bonds, although single bonds are preferred. The length of the chain is not critical and may vary widely, depending on the desired relationship between the molecular weight of the construct and the number of toxin or other groups included on the construct. Best results will generally be obtained with chain lengths ranging from 4 atoms to 1,000 atoms, with preferred chains being those of 6 atoms to 1,000 atoms, and the most preferred being those of from 10 atoms to 100 atoms. The chain thus described is the backbone of the spacer itself, and does not include atoms, groups or side chains bonded to the serially bonded atoms forming the backbone. It does however include "linking groups" at the chain termini joining the chain to G and to T.

In certain embodiments of the invention, the spacer is hydrophilic in character to impart hydrophilicity to the construct. The spacer may thus be any hydrophilic group among those known in the art. Examples are polyalkylene glycols, optionally substituted with groups which may or may not add to their hydrophilic character. Among poly(alkylene glycol)s, polyethylene glycol is a preferred example. Examples of the optional substitutions are alkyl groups, alkoxy groups and hydroxy groups. Unsubstituted polyethylene glycol is particularly preferred. The length of the optionally substituted polyalkylene glycol is not critical and may vary. Selection of the length will be governed by such considerations as achieving the desired molecular weight for the construct and imparting the desired degree of hydrophilic character. In most applications, polyalkylene glycols having molecular weights ranging from about 100 daltons to about 20,000 daltons will provide the best results, with a range of from about 200 daltons to about 10,000 daltons preferred, with about 500 to about 5,000 daltons being particularly preferred. Branched PEGs that include two or more PEG moieties are also of use. Exemplary branched PEG moieties have the same molecular weight as the linear PEGs.

In embodiments of the invention in which the spacer provides *in vivo* cleavability to the construct, the spacer may contain any of a variety of groups as part of its chain which will cleave *in vivo* at a rate which is enhanced relative to that of constructs which lack such groups. Accelerated rates of cleavage enhance the rates of removal of potentially toxic species from the body and/or enhance the activation of less active "prodrug" forms of the toxin when the construct reaches its target, and therefore lower the systemic toxicity of the toxin. Accelerated cleavage rates further permit the administration of higher concentrations of the toxin, thereby increasing the therapeutic efficacy of the treatment regimen. While the degree of cleavage rate enhancement is not critical to the invention, preferred examples of these spacers are those in which at least about 10% of the cleavable groups are cleaved *in vivo* within 24 hours of administration, most preferably at least about 50%.

Exemplary cleavable groups include ester linkages and disulfide linkages. Other structures of use as cleavable linkers include cathepsin cleavable peptides (Dubowchik et al., *Bioconjug Chem.* 13(4):855-69 (2002)), ubiquitinized linkers (Rivett et al., *Arch Biochem Biophys.* 268(1): 1-8 (1989)), polysaccharides and heparinoids. Still further structures of use as cleavable linkers include metabolic intermediates and sucrose analogs. Hydrolytically unstable linkers, *e.g.* acontityl, that are degraded/cleaved off at acidic pH (e.g., pH 5 of lysosome) are also of use as cleavable linkers.

In another embodiment, the protein includes a GlcNAc attached to an Asn of a peptide, wherein the Asn is in or near the cleavage site. The peptide is delivered to the lysosome, where a lysosomal protein cleaves off GlcNAc to expose the cleavage site, thereby delivering the toxin specifically to the lysosome. A variation on the this motif is the use of an O-GalNAc linker to attach the toxin or other moiety to the peptide.

In further embodiments of the invention, the linker is cleaved by the action of one or more enzyme, such as lysosomal hydrolases (Gillelesen et al., *Onkologie* 25(6):534-9 (2002)), lipid phosphatases, acetyl esterases (Derewenda et al., *Biochim Biophys Acta.* 23: 1441(2-3): 229-36 (1999)), sulfohydrolases (i.e. Neiman Pick disease, etc.), endosulfatases (Ai et al., *J Cell Biol.* 21: 162(2): 341-51 (2003)), proteases, lysosomal acid maltases, endo- and exo-glycosidases, for use with linker arms that include various sugars, glycans, glycolipids, oligosaccharides, etc., or endoamylase.

In further embodiments of the invention, the spacer imparts a hydrophilic character to the construct and includes a cleavable group as referred to above.

Exemplary spacer groups of use in the compounds of the invention include, but are not limited to, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl moieties. A presently preferred spacer includes a poly(ethylene glycol) moiety within its framework.

The toxin species represented by the symbol T includes any toxin useful as a therapeutic agent that can be attached to a spacer, sugar or nucleotide sugar. The toxins may be toxic towards host-derived cells (*e.g.* neoplasia) and/or to pathogenic cells that cause infection. Toxins of use in the present invention include known toxins which have been modified or derivatized in any of a variety of ways to achieve a functional group permitting attachment to another moiety. Many drugs and toxins are known which have a cytotoxic effect on neoplasia and on pathogenic microbes that may infect a subject. They can be found in any of the readily available art-recognized compendia of drugs and toxins, such as the Merck Index and the like. Any such antibiotic or cytotoxic drug can be conjugated to an antibody or antibody composite to form a therapeutic agent according to the present invention, and the use of such a conjugate to improve the targeting of an antibiotic or cytotoxic drug to a focus of disease, so as to increase its effective concentration at the site, is a part of the present invention.

The toxin may be obtained from essentially any source; it may be synthetic or a natural product isolated from a selected source, *e.g.*, a plant, bacterial, insect, mammalian or fungal source. The toxin may also be a synthetically modified natural product or an analogue

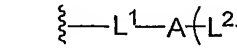
of a natural product. Frequently used plant toxins are divided into two classes: (1) holotoxins (or class II ribosome inactivating proteins), such as ricin, abrin, mistletoe lectin, and modeccin, and (2) hemitoxins (class I ribosome inactivating proteins), such as pokeweed antiviral protein (PAP), saporin, Bryodin 1, bouganin, and gelonin. Commonly used bacterial toxins include diphtheria toxin (DT) and Pseudomonas exotoxin (PE). Kreitman, *Current Pharmaceutical Biotechnology* **2**:313-325 (2001). The toxin may also be an antibody or other peptide.

The compounds and methodology of this invention are also applicable to the therapeutic treatment of infections by conjugating the antibody to a modified sugar bearing an antibiotic or cytotoxic drug or toxin. Such antibiotic or cytotoxic drugs, including, e.g., tetracyclines, chloramphenicol, piperazine, chloroquine, diaminopyridines, metroniazide, isoniazide, rifampins, streptomycins, sulfones, erythromycin, polymixins, nystatin, amphotericins, 5-fluorocytosine, 5-iodo-2'deoxyuridine, 1-adamantanamine, adenine arabinoside, amanitins and azidothymidine (AZT), are preferred for coupling to appropriate specific antibodies/fragments and antibody/fragment composites. Various other potential antibiotic/cytotoxic agents for use in this invention are listed in Goodman et al., "The Pharmacological Basis of Therapeutics," Sixth Edition, A. G. Gilman et al, eds., Macmillan Publishing Co., New York, 1980, showing general art awareness. Various conditions appropriate and desirable for targeting drugs to specific target sites have been reviewed e.g. by Trouet et al., in Targeting of Drugs; G. Gregoriadis et al., eds., Plenum Press, New York and London, 1982, pp. 19-30, showing clinical knowledge of how such targeting benefits patients suffering from infectious lesions. Other exemplary toxins are shown in **FIG. 11**.

Researchers have found that the maximum number of diagnostic or therapeutic moieties that can be directly linked to an antibody is limited by the number of modifiable sites on the antibody molecule and the loss of immunoreactivity of the antibody caused by "over-conjugation". For example, Kulkarni *et al.*, *Cancer Research* **41**: 2700-2706 (1981), have reported that there is a limit to the number of drug molecules that can be incorporated into an antibody without significantly decreasing antigen-binding activity. Kulkarni *et al.*, found that the highest incorporation obtained for methotrexate was about ten methotrexate molecules per molecule of antibody, and that attempts to increase the drug-antibody molar ratio over about ten decreased the yield of immunoconjugate and damaged antibody activity. Kanellos *et al.*, JNCI **75**:319-329 (1985), have reported similar results.

Thus, in another embodiment, the invention provides compounds in which the spacer moiety is composed of subunits, such as multiple spacer moieties, and includes one or

more "amplifying moieties". An amplifying moiety conjugates multiple copies of the toxin to a single site on an antibody. An exemplary compound according to this motif has the general formula:



wherein L^1 is a bond or a spacer moiety covalently joining G to A; A is an amplifier moiety and L^2 is a bond or a spacer moiety covalently joining A to two or more T moieties, and v is a number from 2-1000, with a preferred number being selected from 10-200, and the most preferred number being selected from 20-100. The "amplifier" is a multifunctional group or backbone providing a multitude of attachment sites for spacer groups, toxins or spacer-group-toxin conjugates.

The group A of the formula above, optionally represents an oligomeric or polymeric group which is non-toxic and, at most, minimally antigenic. The amplifier is sufficiently functionalized to permit attachment thereto of a multitude of spacer groups, through covalent linkages. Examples of amplifiers include poly(amino acids), polysaccharides, dendrimers, and derivatized analogs of these groups of compounds, and polymers in general. The term "polymer" as used herein encompasses oligomers. More narrowly defined classes include linear polypeptides and oligopeptides of both essential and nonessential amino acids, including lysine, ornithine and glutamic acid, for example, and any other polypeptides and oligopeptides which have one or more terminal amino groups and are available in the desired molecular weights in narrow ranges. A further class is that of branched synthetic oligopeptides and polypeptides, such as branched dendritic polymers of amino acids such as lysine, the dendritic polymers being readily synthesized in a controlled manner using conventional techniques to yield a controlled number of functional groups. Polysaccharides such as dextrans, starches, and celluloses are a still further class, and simple non-biological polymers such as polyethyleneimine are yet a further class. Derivatized analogs of the polymers of these classes include the polymers modified to contain selected functional groups to permit formation of the linker groups referred to above. An example is poly(aminopropyl)dextran. See, for example, Mann *et al.*, *Bioconjugate Chemistry*, **3**: 154-157 (1992).

A "dendritic polymer" is a polymer exhibiting regular dendritic branching, formed by the sequential or generational addition of branched layers to or from a core. The term dendritic polymer encompasses "dendrimers," which are characterized by a core, at least one interior branched layer, and a surface branched layer. A "dendron" is a species of

dendrimer having branches emanating from a focal point which is or can be joined to a core, either directly or through a linking moiety to form a dendrimer. Many dendrimers comprise two or more dendrons joined to a common core. However, the term dendrimer is used broadly to encompass a single dendron.

Dendritic polymers include, but are not limited to, symmetrical and unsymmetrical branching dendrimers, cascade molecules, arborols, and the like, though the most preferred dendritic polymers are dense star polymers. The well-known PAMAM dense star dendrimers are symmetric, in that the branch arms are of equal length. The branching occurs at a terminal -NH group on a preceding generation branch. In contrast, lysine-based dendrimers are unsymmetrical in that the branch arms are of a different length. One branch occurs at the epsilon nitrogen of the lysine molecule, while another branch occurs at the alpha nitrogen, adjacent to the reactive carboxy group which attaches the branch to a previous generation branch.

Even though not formed by regular sequential addition of branched layers, hyperbranched polymers, e.g., hyperbranched polyols, may be equivalent to a dendritic polymer where the branching pattern exhibits a degree of regularity approaching that of a dendrimer.

In an exemplary embodiment, a polyamidoamine dendrimer is used as the amplifier. Dendrimer molecules of a suitable type can be prepared, for example, by the method of Tomalia et al., *Angew. Chem. Int. Ed. Engl.* 29:138-175 (1990). Dendrimers prepared by this method exhibit uniform size, shape and charge, and carry a known number of primary amine groups on the surface of the molecule, all of which may be used for conjugation purposes. Polyamidoamine dendrimers also bear tertiary amine groups which will be protonated in aqueous solution at physiological pH, conferring aqueous solubility on the carrier molecule.

Dendritic polymers also encompass surface modified dendrimers. For example, the surface of a PAMAM dendrimer may be modified by the addition of an amino acid, e.g., lysine or arginine.

A polypeptide carrier can be also used. At least some of the amino acids are preferably lysine residues or glutamate or aspartate residues. The pendant amines of lysine residues and pendant carboxylates of glutamine and aspartate are convenient for attaching a toxin, spacer or toxin-spacer conjugate. Examples of suitable polypeptide carriers include polylysine, polyglutamic acid, polyaspartic acid, copolymers thereof, and mixed polymers of

these amino acids and others, e.g., serines, to confer desirable solubility properties on the resultant loaded carrier and immunoconjugate.

The antibody component of the conjugate can include whole antibodies, antibody fragments, or subfragments. Use of the term "antibody" herein will be understood to embrace whole antibodies, antibody fragments and subfragments and thus to be equivalent to the term "antibody/fragment" which is used interchangeably therefor in this discussion, unless otherwise noted. Antibodies can be whole immunoglobulin (IgG) of any class, e.g., IgG, IgM, IgA, IgD, IgE, chimeric antibodies or hybrid antibodies with dual or multiple antigen or epitope specifities, or fragments, *e.g.*, $F(ab')_2$, Fab', Fab and the like, including hybrid fragments, and additionally includes any immunoglobulin or any natural, synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex.

The antibody component of the conjugate of the invention can be a single monospecific antibody reacting with one epitope of a cell or its antigen. In such a case, it is preferable for the antibody to bind to an epitope that is different and separate from epitopes to which the patient's own antibodies bind, avoiding the problem of blocking due to saturation of the antigen with native antibodies, and consequent inhibition of targeting.

Alternatively, the antibody component can be polyspecific, *i.e.*, it can include a plurality of antibodies that bind to a plurality of epitopes. The polyspecific antibody component can be a polyclonal antiserum, preferably affinity purified, from an animal which has been challenged with an immunogenic form of the antigen and stimulated to produce a plurality of specific antibodies against the antigen. Another alternative is to use an "engineered polyclonal" mixture, which is a mixture of monoclonal antibodies with a defined range of epitopic specificities.

In both types of polyclonal mixtures, it can be advantageous to chemically link polyspecific antibodies together to form a single polyspecific molecule capable of binding to any of several epitopes. Conjugation of such a polyspecific targeting molecule with a therapeutic agent increases the likelihood that the agent will reach the locus of disease, thereby increasing the target to non-target ratio and the efficacy of the conjugate. One way of effecting such a linkage is to make bivalent $F(ab')_2$ hybrid fragments by mixing two different $F(ab')_2$ fragments produced, e.g., by pepsin digestion of two different antibodies, reductive cleavage to form a mixture of Fab' fragments, followed by oxidative reformation of the disulfide linkages to produce a mixture of $F(ab')_2$ fragments including hybrid fragments containing a Fab' portion specific to each of the original antigens. Methods of preparing such

hybrid antibody fragments are disclosed in Feteanu, "Labeled Antibodies in Biology and Medicine" pages 321-323 (McGraw-Hill Int. Bk. Co., New York et al, 1978); Nisonoff et al, *Arch Biochem. Biophys.*, 93, 470 (1961); and Hammerling et al, *J. Exp. Med.*, 128, 1461 (1968); and in U.S. Pat. No. 4,331,647.

Other methods are known in the art to make bivalent fragments that are entirely heterospecific, e.g., use of bifunctional linkers to join cleaved fragments. Recombinant molecules are known that incorporate the light and heavy chains of an antibody, e.g., according to the method of Boss et al., U.S. Pat. No. 4,816,397. Analogous methods of producing recombinant or synthetic binding molecules having the characteristics of antibodies are included in the invention. More than two different monospecific antibodies or antibody fragments can be linked using various linkers known in the art.

The immunological profile of the substantially monospecific, preferably monoclonal, antibodies used to make polyspecific conjugates useful in the present invention can be adjusted to ensure optimal binding to the pathogen or its antigens by mixing the antibody specificities for different antigens and their epitopes in particular cases of infections, as well as of binding constants for the target epitopes, so as to fine tune the selectivity and targeting efficiency of the reagent according to the invention.

Hybridoma-derived monoclonal antibodies (human, monkey, rat, mouse, or the like) are also suitable for use in the present invention and have the advantage of high specificity. They are readily prepared by what are now generally considered conventional procedures for immunization of mammals with an immunogenic antigen preparation, fusion of immune lymph or spleen cells, with an immortal myeloma cell line, and isolation of specific hybridoma clones. More unconventional methods of preparing monoclonal antibodies are not excluded, such as interspecies fusions and genetic engineering manipulations of hypervariable regions, since it is primarily the antigen specificity of the antibodies that affects their utility in the present invention. Human lymphocytes can be fused with a human myeloma cell line to produce antibodies with particular specificities, preferably to epitopes which are not masked by circulating antibodies to the major antigenic sites on the pathogen.

A therapeutic agent according to the invention can comprise bispecific, trispecific or, more generally, polyspecific antibody/fragment conjugates.

As discussed above, the invention provides methods of preparing conjugates of glycosylated and unglycosylated peptides. The conjugates are formed between peptides and diverse species such as water-soluble polymers, therapeutic moieties, diagnostic

moieties, targeting moieties and the like. Also provided are conjugates that include two or more peptides linked together through a linker arm, i.e., multifunctional conjugates. The multi-functional conjugates of the invention can include two or more copies of the same peptide or a collection of diverse peptides with different structures and/or properties.

The conjugates of the invention are formed by the enzymatic attachment of a modified sugar to the glycosylated or unglycosylated peptide. The modified sugar, when interposed between the peptide and the modifying group on the sugar becomes what is referred to herein as "an intact glycosyl linking group." Using the exquisite selectivity of enzymes, such as glycosyltransferases, the present method provides peptides that bear a desired group at one or more specific locations. Thus, according to the present invention, a modified sugar is attached directly to a selected locus on the peptide chain or, alternatively, the modified sugar is appended onto a carbohydrate moiety of a glycopeptide. Peptides in which modified sugars are bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone are also within the scope of the present invention.

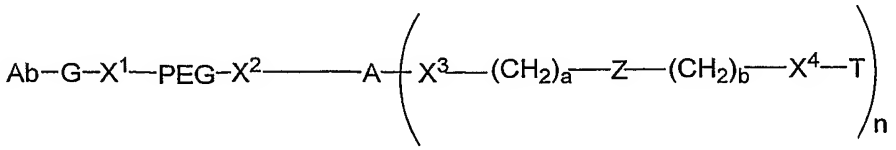
In contrast to known chemical and enzymatic peptide elaboration strategies, the methods of the invention, make it possible to assemble peptides and glycopeptides that have a substantially homogeneous derivatization pattern; the enzymes used in the invention are generally selective for a particular amino acid residue or combination of amino acid residues of the peptide. The methods are also practical for large-scale production of modified peptides and glycopeptides. Thus, the methods of the invention provide a practical means for large-scale preparation of glycopeptides having preselected uniform derivatization patterns. The methods are particularly well suited for modification of antibodies, including but not limited to, glycopeptides that are incompletely glycosylated during production in cell culture (*e.g.*, mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or prokaryotic cells) or transgenic plants or animals.

In another embodiment, the invention provides a peptide conjugate having a formula selected from:

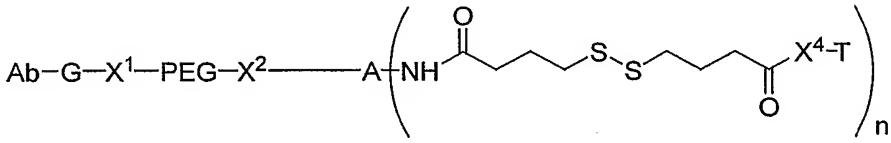
$$Ab-G-(PEG)_m-(toxin)_w;\ Ab-G-L^1-(amplifier)_m-(L^2-toxin)_n;\ \text{and}\ Ab-G-(L^1)_m-(toxin)_n$$

in which PEG is a straight- or branched-chain poly(ethylene glycol); m is an integer from 1 to 6; and n is an integer from 1 to 1,000. In those embodiments in which linear PEG is used, w = m; when a branched PEG is used, w = (m X number of branches). For example, if a branched PEG with two branches is used, w = (1x2) = $(toxin)_2$.

In an exemplary embodiment, the invention provides a conjugate having the formula:



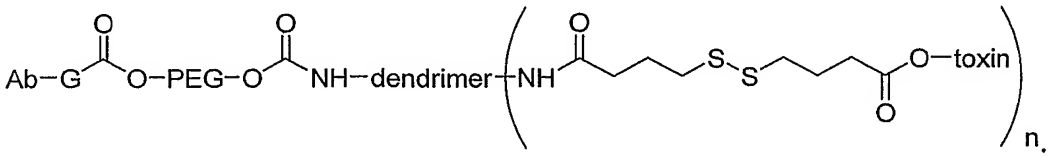
In still a further exemplary embodiment, the invention provides a peptide conjugate having the formula:



In the formula above, the symbols X^1, X^2 and X^4 represent inert linking groups which serve to join the various groups together. The nature of these linking groups is not critical, and their selection will be largely a matter of convenience as determined by the means of synthesis of the construct. The term "inert" in this context means essentially non-toxic, non-immunogenic, and stable with respect to cleavage or dissociation over the typical period of time required for use of the construct in a clinical or diagnostic procedure. Examples of inert linking groups useful for this purpose are alkylamino or aminoalkyl groups such as $(CH_2)_q$-NH, NH-$(CH_2)_q$, carbamoyl groups such as NH-C(O)-O, O-C(O)-NH, alkylcarbamoyl or carbanoylalkyl groups such as $(CH_2)_q$-NH-C(O)-O, O-C(O)-NH-$(CH_2)_q$, C(O)-O, O-C(O), $(CH_2)_q$-NH-C(O), C(O)-NH-$(CH_2)_q$, C(O)NH, NHC(O), NH-C(S), and C(S)-NH.

The symbol q in these groups may vary, but in most cases will generally range from 0 to 20, with 2 to 6 preferred, and 2 to 3 particularly preferred. The symbol n in these groups may also vary and will generally range from 1 to 1,000, with 2 to 100 being preferred and 4 to 10 being particularly preferred.

In yet another exemplary embodiment, the invention provides a peptide conjugate that combines the intact glycosyl linker, a dendrimer and a PEG moiety. A representative compound according to this embodiment has the formula:



Many further alternatives to these structures exist. For example, to produce constructs containing cleavable esters in the spacers without PEG, an amine- or hydroxyl-containing amplifying polymer can be derivatized to produce carboxylic acid groups as the functional groups. This is readily achieved by reacting the polymer with maleic, succinic or glutaric anhydride using established procedures. A derivatized toxin or spacer to combine with the derivatized polymer can be formed by reacting a species bearing an isothiocyanate group with an amino alcohol, *e.g.*, $HO(CH_2)_qNH_2$, to place a terminal hydroxyl group on the toxin, spacer arm or toxin-spacer-arm conjugate. The carboxylic acid group on the derivatized polymer can then be activated by conventional methods using such agents as dicyclohexylcarbodiimide or carbonyl-diimidazole, and reacted with the derivatized species to achieve the ester linkage. The section of the construct between the amplifying polymer and the toxin serves as a spacer, and the length of the spacer is determined by the number of CH_2 groups in the amino alcohol used to derivative the ligand.

In an alternate scheme which produces a reverse ester, the toxin is derivatized with an aminocarboxylic acid, $HO_2C(CH_2)_q NH_2$, rather than an amino alcohol. The resulting carboxylic acid-derivatized toxin is then activated with dicyclohexylcarbodiimide or carbonyldiimidazole and coupled directly to a hydroxyl-containing amplifying polymer.

In either of these two schemes, a selected fraction of the amine or hydroxyl groups which are native to the amplifying polymer can be protected if desired, to avoid interference with the coupling reactions. This is readily achieved by conventional methods Synthesis of the compounds of the invention are by techniques which are well known to those of skill in the art.

The present invention also provides sugars, activated sugars and sugar nucleotides that are modified with a toxin. The toxin can be attached directly to the sugar subunit or it is optionally attached to a spacer arm, spacer arm-amplifier construct, spacer arm-amplifier-spacer arm construct or the like. The compounds in this aspect of the invention have the following general formula:

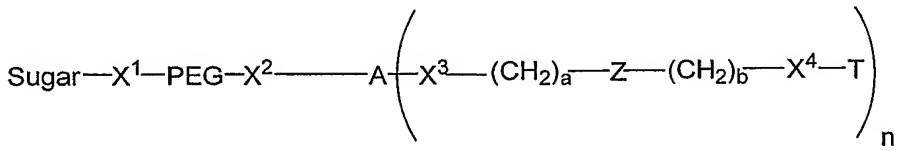
S–L—T

wherein S is a sugar, or an activated sugar, or a nucleotide sugar; L is a bond or a spacer moiety (optionally including an amplifier) covalently joining S to T; and
T is a toxin moiety.

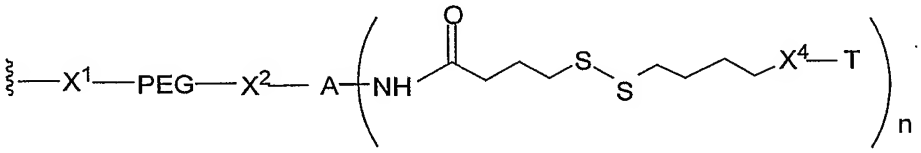
Exemplary modified sugars according to this aspect of the invention include::

$S-(PEG)_m-(toxin)_w$; $S-L^1-(amplifier)_m-(L^2-toxin)_n$; and $S-(L^1)_m-(toxin)_n$.

In another exemplary embodiment, the invention provides a modified sugar according to the formula:

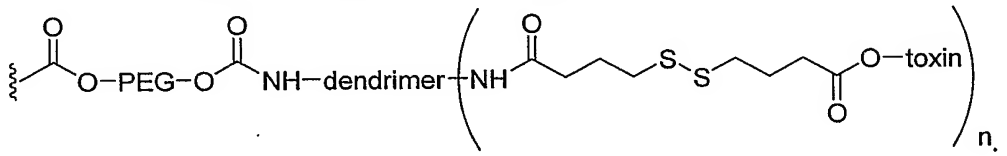


In another exemplary embodiment, the invention provides a modified sugar including a moiety having the formula:



wherein X^1, X^2 and X^4 are inert linking groups and are members selected from the group consisting of $(CH_2)_q$-NH, NH-$(CH_2)_q$, NH-C(O)-O, O-C(O)-NH, $(CH_2)_q$-NH-C(O)-O, O-C(O)-NH-$(CH_2)_q$, C(O)-O, O-C(O), $(CH_2)_q$-NH-C(O), C(O)-NH-$(CH_2)_q$, NH-C(S), and C(S)-NH.

Yet another exemplary modified sugar of the invention has the formula:



The components of the generic structures above are substantially similar to those for the peptide conjugates discussed hereinabove, with the exception that L joins S to T. The identities of the indices "m", "n", "q" and "w" are identified to those discussed above.

Modified Sugars

Modified glycosyl donor species ("modified sugars") are preferably selected from modified sugar nucleotides, activated modified sugars and modified sugars that are simple saccharides that are neither nucleotides nor activated. Any desired carbohydrate structure can be added to a peptide using the methods of the invention. Typically, the structure will be a monosaccharide, but the present invention is not limited to the use of modified monosaccharide sugars; oligosaccharides and polysaccharides are useful as well.

The modifying group is attached to a sugar moiety by enzymatic means, chemical means or a combination thereof, thereby producing a modified sugar. The sugars are substituted at any position that allows for the attachment of the modifying moiety, yet

which still allows the sugar to function as a substrate for the enzyme used to ligate the modified sugar to the peptide. In a preferred embodiment, when sialic acid is the sugar, the sialic acid is substituted with the modifying group at either the 9-position on the pyruvyl side chain or at the 5-position on the amine moiety that is normally acetylated in sialic acid.

In certain embodiments of the present invention, a modified sugar nucleotide is utilized to add the modified sugar to the peptide. Exemplary sugar nucleotides that are used in the present invention in their modified form include nucleotide mono-, di- or triphosphates or analogs thereof. In a preferred embodiment, the modified sugar nucleotide is selected from a UDP-glycoside, CMP-glycoside, or a GDP-glycoside. Even more preferably, the modified sugar nucleotide is selected from a UDP-galactose, UDP-galactosamine, UDP-glucose, UDP-glucosamine, GDP-mannose, GDP-fucose, CMP-sialic acid, or CMP-NeuAc. N-acetylamine derivatives of the sugar nucletides are also of use in the method of the invention.

The invention provides methods for synthesizing a modified peptide using a modified sugar, *e.g.*, modified-galactose, -fucose, and –sialic acid. When a modified sialic acid is used, either a sialyltransferase or a trans-sialidase (for α2,3-linked sialic acid only) can be used in these methods.

In other embodiments, the modified sugar is an activated sugar. Activated modified sugars, which are useful in the present invention are typically glycosides which have been synthetically altered to include an activated leaving group. As used herein, the term "activated leaving group" refers to those moieties, which are easily displaced in enzyme-regulated nucleophilic substitution reactions. Many activated sugars are known in the art. See, for example, Vocadlo et al., In CARBOHYDRATE CHEMISTRY AND BIOLOGY, Vol. 2, Ernst *et al.* Ed., Wiley-VCH Verlag: Weinheim, Germany, 2000; Kodama *et al.*, *Tetrahedron Lett.* **34**: 6419 (1993); Lougheed, *et al., J. Biol. Chem.* **274**: 37717 (1999)).

Examples of activating groups (leaving groups) include fluoro, chloro, bromo, tosylate ester, mesylate ester, triflate ester and the like. Preferred activated leaving groups, for use in the present invention, are those that do not significantly sterically encumber the enzymatic transfer of the glycoside to the acceptor. Accordingly, preferred embodiments of activated glycoside derivatives include glycosyl fluorides and glycosyl mesylates, with glycosyl fluorides being particularly preferred. Among the glycosyl fluorides, α-galactosyl fluoride, α-mannosyl fluoride, α-glucosyl fluoride, α-fucosyl fluoride, α-xylosyl fluoride, α-sialyl fluoride, α-N-acetylglucosaminyl fluoride, α-N-acetylgalactosaminyl fluoride, β-

galactosyl fluoride, β-mannosyl fluoride, β-glucosyl fluoride, β-fucosyl fluoride, β-xylosyl fluoride, β-sialyl fluoride, β-N-acetylglucosaminyl fluoride and β-N-acetylgalactosaminyl fluoride are most preferred.

By way of illustration, glycosyl fluorides can be prepared from the free sugar by first acetylating the sugar and then treating it with HF/pyridine. This generates the thermodynamically most stable anomer of the protected (acetylated) glycosyl fluoride (*i.e.*, the α-glycosyl fluoride). If the less stable anomer (*i.e.*, the β-glycosyl fluoride) is desired, it can be prepared by converting the peracetylated sugar with HBr/HOAc or with HCl to generate the anomeric bromide or chloride. This intermediate is reacted with a fluoride salt such as silver fluoride to generate the glycosyl fluoride. Acetylated glycosyl fluorides may be deprotected by reaction with mild (catalytic) base in methanol (*e.g.* NaOMe/MeOH). In addition, many glycosyl fluorides are commercially available.

Other activated glycosyl derivatives can be prepared using conventional methods known to those of skill in the art. For example, glycosyl mesylates can be prepared by treatment of the fully benzylated hemiacetal form of the sugar with mesyl chloride, followed by catalytic hydrogenation to remove the benzyl groups.

In a further exemplary embodiment, the modified sugar is an oligosaccharide having an antennary structure. In a preferred embodiment, one or more of the termini of the antennae bear the modifying moiety. When more than one modifying moiety is attached to an oligosaccharide having an antennary structure, the oligosaccharide is useful to "amplify" the modifying moiety; each oligosaccharide unit conjugated to the peptide attaches multiple copies of the modifying group to the peptide. The general structure of a typical chelate of the invention as set forth in the drawing above, encompasses multivalent species resulting from preparing a conjugate of the invention utilizing an antennary structure. Many antennary saccharide structures are known in the art, and the present method can be practiced with them without limitation.

Exemplary modifying groups are discussed below. The modifying groups can be selected for one or more desirable property. Exemplary properties include, but are not limited to, enhaced pharmacokinetics, enhanced pharmacodynamics, improved biodistribution, providing a polyvalent species, improved water solubility, enhanced or diminished lipophilicity, and tissue targeting.

Water-Soluble Polymers

As discussed above, the hydrophilicity of a selected peptide is enhanced by conjugation with polar molecules such as amine-, ester-, hydroxyl- and polyhydroxyl-containing molecules. Representative examples include, but are not limited to, polylysine, polyethyleneimine, poly(ethyleneglycol) and poly(propyleneglycol). Preferred water-soluble polymers are essentially non-fluorescent, or emit such a minimal amount of fluorescence that they are inappropriate for use as a fluorescent marker in an assay. Moreover, it is generally preferred to use polymers that are not naturally occurring sugars. An exception to this preference is the use of an otherwise naturally occurring sugar that is modified by covalent attachment of another entity (*e.g.*, poly(ethylene glycol), poly(propylene glycol), biomolecule, therapeutic moiety, diagnostic moiety, *etc.*). In another exemplary embodiment, a sugar moiety is conjugated to a poly(ethylene glycol)-toxin construct and the sugar-PEG-toxin cassette is subsequently conjugated to a peptide via a method of the invention.

Methods and chemistry for activation of water-soluble polymers and saccharides as well as methods for conjugating saccharides and polymers to various species are described in the literature. Commonly used methods for activation of polymers include activation of functional groups with cyanogen bromide, periodate, glutaraldehyde, biepoxides, epichlorohydrin, divinylsulfone, carbodiimide, sulfonyl halides, trichlorotriazine, *etc.* (*see*, R. F. Taylor, (1991), PROTEIN IMMOBILISATION. FUNDAMENTALS AND APPLICATIONS, Marcel Dekker, N.Y.; S. S. Wong, (1992), CHEMISTRY OF PROTEIN CONJUGATION AND CROSSLINKING, CRC Press, Boca Raton; G. T. Hermanson *et al.*, (1993), IMMOBILIZED AFFINITY LIGAND TECHNIQUES, Academic Press, N.Y.; Dunn, R.L., *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

Many water-soluble polymers are known to those of skill in the art and are useful in practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (e.g., dextran, amylose, hyalouronic acid, poly(sialic acid), heparans, heparins, etc.); poly (amino acids); nucleic acids; synthetic polymers (e.g., poly(acrylic acid), poly(ethers), e.g., poly(ethylene glycol); peptides, proteins, and the like. The present invention may be practiced with any water-soluble polymer with the sole limitation that the polymer must include a point at which the remainder of the conjugate can be attached.

Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No.

5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for conjugation between activated polymers and peptides, *e.g.* Coagulation Factor VIII (WO 94/15625), haemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese *at al., App. Biochem. Biotech.* **11**: 141-45 (1985)).

Preferred water-soluble polymers are those in which a substantial proportion of the polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are "homodisperse."

The present invention is further illustrated by reference to a poly(ethylene glycol) conjugate. Several reviews and monographs on the functionalization and conjugation of PEG are available. *See,* for example, Harris, M*acronol. Chem. Phys.* **C25**: 325-373 (1985); Scouten, *Methods in Enzymology* **135**: 30-65 (1987); Wong *et al.*, *Enzyme Microb. Technol.* **14**: 866-874 (1992); Delgado *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **9**: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* **6**: 150-165 (1995); and Bhadra, *et al.*, *Pharmazie*, **57**:5-29 (2002).

The poly(ethylene glycol) useful in forming the conjugate of the invention is either linear or branched.

In another embodiment, analogous to those discussed above, the modifying group is a water-insoluble polymer, rather than a water-soluble polymer. The glyco-conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic peptide in a controlled manner. Polymeric drug delivery systems are known in the art. *See*, for example, Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991. Those of skill in the art will appreciate that substantially any known drug delivery system is applicable to the conjugates of the present invention.

Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes, polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate),

poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly (ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronics and polyvinylphenol and copolymers thereof.

Synthetically modified natural polymers of use in the glyco-conjugates of the invention include, but are not limited to, alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, and nitrocelluloses. Particularly preferred members of the broad classes of synthetically modified natural polymers include, but are not limited to, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxymethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, and polymers of acrylic and methacrylic esters and alginic acid.

These and the other polymers discussed herein can be readily obtained from commercial sources such as Sigma Chemical Co. (St. Louis, MO.), Polysciences (Warrenton, PA.), Aldrich (Milwaukee, WI.), Fluka (Ronkonkoma, NY), and BioRad (Richmond, CA), or else synthesized from monomers obtained from these suppliers using standard techniques.

Representative biodegradable polymers of use in the conjugates of the invention include, but are not limited to, polylactides, polyglycolides and copolymers thereof, poly(ethylene terephthalate), poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), poly(lactide-co-glycolide), polyanhydrides, polyorthoesters, blends and copolymers thereof. Of particular use are compositions that form gels, such as those including collagen, pluronics and the like.

The polymers of use in the invention include "hybrid' polymers that include water-insoluble materials having within at least a portion of their structure, a bioresorbable molecule. An example of such a polymer is one that includes a water-insoluble copolymer, which has a bioresorbable region, a hydrophilic region and a plurality of crosslinkable functional groups per polymer chain.

For purposes of the present invention, "water-insoluble materials" includes materials that are substantially insoluble in water or water-containing environments. Thus, although certain regions or segments of the copolymer may be hydrophilic or even water-soluble, the polymer molecule, as a whole, is not substantially soluble in water.

For purposes of the present invention, the term "bioresorbable molecule" includes a region that is capable of being metabolized or broken down and resorbed and/or eliminated through normal excretory routes by the body. Such metabolites or break down products are preferably substantially non-toxic to the body.

The bioresorbable region may be either hydrophobic or hydrophilic, so long as the copolymer composition as a whole is not rendered water-soluble. Thus, the bioresorbable region is selected based on the preference that the polymer, as a whole, remains water-insoluble. Accordingly, the relative properties, *i.e.*, the kinds of functional groups contained by, and the relative proportions of the bioresorbable region, and the hydrophilic region are selected to ensure that useful bioresorbable compositions remain water-insoluble.

Exemplary resorbable polymers include, for example, synthetically produced resorbable block copolymers of poly(α-hydroxy-carboxylic acid)/poly(oxyalkylene, (*see*, Cohn *et al.*, U.S. Patent No. 4,826,945). These copolymers are not crosslinked and are water-soluble so that the body can excrete the degraded block copolymer compositions. *See*, Younes *et al.*, *J Biomed. Mater. Res.* **21**: 1301-1316 (1987); and Cohn *et al.*, *J Biomed. Mater. Res.* **22**: 993-1009 (1988).

Presently preferred bioresorbable polymers include one or more components selected from poly(esters), poly(hydroxy acids), poly(lactones), poly(amides), poly(ester-amides), poly (amino acids), poly(anhydrides), poly(orthoesters), poly(carbonates), poly(phosphazines), poly(phosphoesters), poly(thioesters), polysaccharides and mixtures thereof. More preferably still, the biosresorbable polymer includes a poly(hydroxy) acid component. Of the poly(hydroxy) acids, polylactic acid, polyglycolic acid, polycaproic acid, polybutyric acid, polyvaleric acid and copolymers and mixtures thereof are preferred.

In addition to forming fragments that are absorbed *in vivo* ("bioresorbed"), preferred polymeric coatings for use in the methods of the invention can also form an excretable and/or metabolizable fragment.

Higher order copolymers can also be used in the present invention. For example, Casey *et al.*, U.S. Patent No. 4,438,253, which issued on March 20, 1984, discloses tri-block copolymers produced from the transesterification of poly(glycolic acid) and an hydroxyl-ended poly(alkylene glycol). Such compositions are disclosed for use as resorbable monofilament sutures. The flexibility of such compositions is controlled by the incorporation

of an aromatic orthocarbonate, such as tetra-p-tolyl orthocarbonate into the copolymer structure.

Other polymers based on lactic and/or glycolic acids can also be utilized. For example, Spinu, U.S. Patent No. 5,202,413, which issued on April 13, 1993, discloses biodegradable multi-block copolymers having sequentially ordered blocks of polylactide and/or polyglycolide produced by ring-opening polymerization of lactide and/or glycolide onto either an oligomeric diol or a diamine residue followed by chain extension with a di-functional compound, such as, a diisocyanate, diacylchloride or dichlorosilane.

Bioresorbable regions of coatings useful in the present invention can be designed to be hydrolytically and/or enzymatically cleavable. For purposes of the present invention, "hydrolytically cleavable" refers to the susceptibility of the copolymer, especially the bioresorbable region, to hydrolysis in water or a water-containing environment. Similarly, "enzymatically cleavable" as used herein refers to the susceptibility of the copolymer, especially the bioresorbable region, to cleavage by endogenous or exogenous enzymes.

When placed within the body, the hydrophilic region can be processed into excretable and/or metabolizable fragments. Thus, the hydrophilic region can include, for example, polyethers, polyalkylene oxides, polyols, poly(vinyl pyrrolidine), poly(vinyl alcohol), poly(alkyl oxazolines), polysaccharides, carbohydrates, peptides, proteins and copolymers and mixtures thereof. Furthermore, the hydrophilic region can also be, for example, a poly(alkylene) oxide. Such poly(alkylene) oxides can include, for example, poly(ethylene) oxide, poly(propylene) oxide and mixtures and copolymers thereof.

Polymers that are components of hydrogels are also useful in the present invention. Hydrogels are polymeric materials that are capable of absorbing relatively large quantities of water. Examples of hydrogel forming compounds include, but are not limited to, polyacrylic acids, sodium carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidine, gelatin, carrageenan and other polysaccharides, hydroxyethylenemethacrylic acid (HEMA), as well as derivatives thereof, and the like. Hydrogels can be produced that are stable, biodegradable and bioresorbable. Moreover, hydrogel compositions can include subunits that exhibit one or more of these properties.

Bio-compatible hydrogel compositions whose integrity can be controlled through crosslinking are known and are presently preferred for use in the methods of the

invention. For example, Hubbell *et al.*, U.S. Patent Nos. 5,410,016, which issued on April 25, 1995 and 5,529,914, which issued on June 25, 1996, disclose water-soluble systems, which are crosslinked block copolymers having a water-soluble central block segment sandwiched between two hydrolytically labile extensions. Such copolymers are further end-capped with photopolymerizable acrylate functionalities. When crosslinked, these systems become hydrogels. The water soluble central block of such copolymers can include poly(ethylene glycol); whereas, the hydrolytically labile extensions can be a poly(α-hydroxy acid), such as polyglycolic acid or polylactic acid. *See*, Sawhney *et al.*, *Macromolecules* **26**: 581-587 (1993).

In another preferred embodiment, the gel is a thermoreversible gel. Thermoreversible gels including components, such as pluronics, collagen, gelatin, hyalouronic acid, polysaccharides, polyurethane hydrogel, polyurethane-urea hydrogel and combinations thereof are presently preferred.

In yet another exemplary embodiment, the conjugate of the invention includes a component of a liposome. Liposomes can be prepared according to methods known to those skilled in the art, for example, as described in Eppstein *et al.*, U.S. Patent No. 4,522,811, which issued on June 11, 1985. For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its pharmaceutically acceptable salt is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

The above-recited microparticles and methods of preparing the microparticles are offered by way of example and they are not intended to define the scope of microparticles of use in the present invention. It will be apparent to those of skill in the art that an array of microparticles, fabricated by different methods, are of use in the present invention.

II. Methods

In addition to the compositions discussed above, the present invention provides methods for preparing glyco-conjugates. Moreover, the invention provides methods

of preventing, curing or ameliorating a disease state by administering a conjugate of the invention to a subject at risk of developing the disease or a subject that has the disease.

Thus, the invention provides a method of forming a glyco-conjugate between a modifying group and a glycosyl-containing compound, *e.g.*, a glycopeptide, or a glycolipid. For clarity of illustration, the invention is illustrated with reference to a conjugate formed between a glycopeptide and an activated modifying group that includes a water-soluble polymer. Those of skill will appreciate that the invention equally encompasses methods of forming conjugates of glycolipids with water-soluble polymers, and forming conjugates between glycopeptides and glycolipids and modifying groups other than water-soluble polymers.

In exemplary embodiments, the conjugate is formed between a water-soluble polymer, a therapeutic moiety, targeting moiety or a biomolecule, and a glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via a glycosyl linking group, which is interposed between, and covalently linked to, both the peptide (directly or through an intervening glycosyl linker) and the modifying group (*e.g.*, water-soluble polymer). The method includes contacting the glycopeptide with an activated modifying group and an enzyme for which the activated modifying group is a substrate. The components of the reaction mixture are combined under conditions appropriate to acylate a selected glycosyl residue on the glycopeptide, thereby preparing the conjugate.

The acceptor peptide is typically synthesized *de novo*, or recombinantly expressed in a prokaryotic cell (*e.g.*, bacterial cell, such as *E. coli*) or in a eukaryotic cell such as a mammalian, yeast, insect, fungal or plant cell. The peptide can be either a full-length protein or a fragment. Moreover, the peptide can be a wild type or mutated peptide. In an exemplary embodiment, the peptide includes a mutation that adds one or more N- or O-linked glycosylation sites to the peptide sequence.

The method of the invention also provides for modification of incompletely glycosylated peptides that are produced recombinantly. Many recombinantly produced glycoproteins are incompletely glycosylated, exposing carbohydrate residues that may have undesirable properties, *e.g.*, immunogenicity, recognition by the RES. The incomplete glycosyl residue can be masked using a water-soluble polymer.

Exemplary peptides that can be modified using the methods of the invention are set forth in Table 1.

Table 1

Hormones and Growth Factors • G-CSF • GM-CSF • M-CSF • TPO • EPO • EPO variants • alpha-TNF • Leptin Enzymes and Inhibitors • t-PA • t-PA variants • Urokinase • Factors VII, VIII, IX, X • Dnase • Glucocerebrosidase • Hirudin • α1 antitrypsin • Antithrombin III Cytokines and Chimeric Cytokines • Interleukin-1 (IL-1), 1B, 2, 3, 4 • Interferon-alpha (IFN-alpha) • IFN-alpha-2b • IFN-beta • IFN-gamma • Chimeric diptheria toxin-IL-2	Receptors and Chimeric Receptors • CD4 • Tumor Necrosis Factor (TNF) receptor • Alpha-CD20 • MAb-CD20 • MAb-alpha-CD3 • MAb-TNF receptor • MAb-CD4 • PSGL-1 • MAb-PSGL-1 • Complement • GlyCAM or its chimera • N-CAM or its chimera Monoclonal Antibodies (Immunoglobulins) • MAb-anti-RSV • MAb-anti-IL-2 receptor • MAb-anti-CEA • MAb-anti-platelet IIb/IIIa receptor • MAb-anti-EGF • MAb-anti-Her-2 receptor Cells • Red blood cells • White blood cells (*e.g.*, T cells, B cells, dendritic cells, macrophages, NK cells, neutrophils, monocytes and the like • Stem cells

Other exemplary peptides that are modified by the methods of the invention include members of the immunoglobulin family (*e.g.*, antibodies, MHC molecules, T cell receptors, and the like), intercellular receptors (*e.g.*, integrins, receptors for hormones or growth factors and the like) lectins, and cytokines (*e.g.*, interleukins). Additional examples include tissue-type plasminogen activator (t-PA), renin, clotting factors such as factors V-XII, bombesin, thrombin, hematopoietic growth factor, colony stimulating factors, viral antigens, complement proteins, α1-antitrypsin, erythropoietin, P-selectin glycopeptide ligand-1 (PSGL-1), granulocyte-macrophage colony stimulating factor, anti-thrombin III, interleukins, interferons, proteins A and C, fibrinogen, herceptin, leptin, glycosidases, HS-glycoprotein, serum proteins (e.g., α-acid glycoprotein, fetuin, α-fetal protein), β2-glycoprotein, among many others. This list of polypeptides is exemplary, not exclusive. The methods are also useful for modifying fusion and chimeric proteins, including, but not limited to, chimeric

proteins that include a moiety derived from an immunoglobulin, such as IgG, or a fragment of an immunoglobin, *e.g.*, FAb (Fc domain). The exemplary peptides provided herein are intended to provide a selection of the peptides with which the present invention can be practiced; as such, they are non-limiting. Those of skill will appreciate that the invention can be practiced using substantially any peptide from any source.

Peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An exemplary N-linkage is the attachment of the modified sugar to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of a carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one sugar (*e.g.*, N-acetylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to the hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although unusual or non-natural amino acids, e.g., 5-hydroxyproline or 5-hydroxylysine may also be used.

Moreover, in addition to peptides, the methods of the present invention can be practiced with other biological structures (*e.g.*, glycolipids, lipids, sphingoids, ceramides, whole cells, and the like, containing a glycosylation site).

Addition of glycosylation sites to a peptide or other structure is conveniently accomplished by altering the amino acid sequence such that it contains one or more glycosylation sites. The addition may also be made by the incorporation of one or more species presenting an –OH group, preferably serine or threonine residues, within the sequence of the peptide (for O-linked glycosylation sites). The addition may be made by mutation or by full chemical synthesis of the peptide. The peptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the peptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) are preferably made using methods known in the art.

In an exemplary embodiment, the glycosylation site is added by shuffling polynucleotides. Polynucleotides encoding a candidate peptide can be modulated with DNA shuffling protocols. DNA shuffling is a process of recursive recombination and mutation,

performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. *See, e.g.*, Stemmer, *Proc. Natl. Acad. Sci. USA* 91:10747-10751 (1994); Stemmer, *Nature* 370:389-391 (1994); and U.S. Patent Nos. 5,605,793, 5,837,458, 5,830,721 and 5,811,238.

The present invention also provides means of adding (or removing) one or more selected glycosyl residues to a peptide, after which a modified sugar is conjugated to at least one of the selected glycosyl residues of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified sugar to a selected glycosyl residue that is either not present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified sugar to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation pattern of a glycopeptide is altered prior to the conjugation of the modified sugar by the removal of a carbohydrate residue from the glycopeptide. *See*, for example WO 98/31826.

Addition or removal of any carbohydrate moieties present on the glycopeptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the polypeptide variant to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin *et al., Arch. Biochem. Biophys.* **259**: 52 (1987) and by Edge *et al., Anal. Biochem.* **118**: 131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al., Meth. Enzymol.* **138**: 350 (1987).

Chemical addition of glycosyl moieties is carried out by any art-recognized method. Enzymatic addition of sugar moieties is preferably achieved using a modification of the methods set forth herein, substituting native glycosyl units for the modified sugars used in the invention. Other methods of adding sugar moieties are disclosed in U.S. Patent No. 5,876,980, 6,030,815, 5,728,554, and 5,922,577.

Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) consensus sites for N-linked glycosylation, and sites for O-linked glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyltransferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulfhydryl groups such as

those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC CRIT. REV. BIOCHEM., pp. 259-306 (1981).

In one embodiment, the invention provides a method for linking two or more peptides through a linking group. The linking group is of any useful structure and may be selected from straight- and branched-chain structures. Preferably, each terminus of the linker, which is attached to a peptide, includes a modified sugar.

In an exemplary method of the invention, two peptides are linked together via a linker moiety that includes a polymeric (*e.g.,* PEG linker). The focus on a PEG linker that includes two glycosyl groups is for purposes of clarity and should not be interpreted as limiting the identity of linker arms of use in this embodiment of the invention.

Methods of Preparing the Conjugates

In addition to the conjugates discussed above, the present invention provides methods for preparing these and other conjugates. Thus, in a further aspect, the invention provides a method of forming a covalent conjugate between a selected moiety and a peptide, *e.g.* antibody. Additionally, the invention provides methods for targeting conjugates of the invention to a particular tissue or region of the body.

In exemplary embodiments, the conjugate is formed between a toxin, and a glycosylated or non-glycosylated antibody. The toxin is conjugated to the peptide via an glycosyl linking group, *e.g.* an intact glycosyl linking group, which is interposed between, and covalently linked to both the peptide and the toxin (or a spacer attached to the toxin). The method includes contacting the peptide with a mixture containing a modified sugar and an enzyme, *e.g.* glycosyltransferase for which the modified sugar is a substrate. The reaction is conducted under conditions appropriate to form a covalent bond between the modified sugar and the peptide. The sugar moiety of the modified sugar is preferably selected from nucleotide sugars, and activated sugars.

Alternatively, the method of the invention provides for contacting either the peptide or toxin with a modified sugar that includes a species bearing a reactive organic functional group, thereby appending the modified sugar to the antibody. When the modified sugar forms a glycosyl linking group with the peptide, the reactive functional group is

subsequently utilized to append additional functionality onto the peptide. For example, a spacer, amplifier, spacer-amplifier conjugate, toxin, or a conjugate of a toxin with one of these species is appended to the peptide via reaction of the reactive functional group.

Peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An exemplary N-linkage is the attachment of the modified sugar to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of a carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one sugar (*e.g.*, N-aceylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to a the hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

The acceptor peptide (glycosylated or non-glycosylated) is typically synthesized *de novo*, or recombinantly expressed in a prokaryotic cell (*e.g.*, bacterial cell, such as *E. coli*) or in a eukaryotic cell such as a mammalian, yeast, insect, fungal or plant cell. The peptide can be either a full-length protein or a fragment. Moreover, the peptide can be a wild type or mutated peptide. In an exemplary embodiment, the peptide includes a mutation that adds one or more consensus glycosylation sites to the peptide sequence.

In addition to forming toxin-antibody conjugates through glycosyl linking groups, the invention also provides for modification of incompletely glycosylated peptides that are produced recombinantly. Many recombinantly produced glycoproteins are incompletely glycosylated, exposing carbohydrate residues that may have undesirable properties, e.g., immunogenicity, recognition by the RES. Employing a modified sugar in a method of the invention, the peptide can be simultaneously further glycosylated and derivatized with, e.g., a water-soluble polymer, therapeutic agent, or the like. The sugar moiety of the modified sugar can be the residue that would properly be conjugated to the acceptor in a fully glycosylated peptide, or another sugar moiety with desirable properties. The glycosylation pattern of the peptide can be altered before or after forming the antibody-toxin conjugate.

The methods of the invention also provide conjugates of glycosylated and unglycosylated peptides with increased therapeutic half-life due to, for example, reduced

clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents can also be used to target a peptide to a particular tissue or cell surface receptor that is specific for the particular targeting agent. Moreover, there is provided a class of peptides that are specifically modified with a therapeutic moiety, *e.g.*, a toxin.

As discussed above, the present invention provides a conjugate between a peptide and a selected moiety. The link between the peptide and the selected moiety includes an intact glycosyl linking group interposed between the peptide and the selected moiety. As discussed above, the selected moiety is a toxin, spacer or toxin-spacer conjugate that can be attached to a saccharide unit, resulting in a "modified sugar" that is recognized by an appropriate transferase enzyme, which appends the modified sugar onto the peptide. The saccharide component of the modified sugar, when interposed between the peptide and a selected moiety, becomes a "glycosyl linking group", *e.g.* an "intact glycosyl linking group." The glycosyl linking group is formed from any mono- or oligo-saccharide that, after modification with a selected moiety, is a substrate for an appropriate transferase.

In addition to providing conjugates that are formed through an enzymatically added glycosyl linking group, the method of preparing the conjugates provides conjugates that are highly homogenous in their derivatization patterns. Using the methods of the invention, it is possible to form peptide conjugates in which essentially all of the modified sugar moieties across a population of conjugates of the invention are attached to multiple copies of a structurally identical amino acid or glycosyl residue. Thus, in an exemplary embodiment, essentially each member of the conjugate population is bound via the glycosyl linking group to a glycosyl residue of the peptide, and each glycosyl residue of the peptide to which the glycosyl linking group is attached has the same structure.

Addition of glycosylation sites to a peptide or other structure is conveniently accomplished by altering the amino acid sequence such that it contains one or more glycosylation sites. The addition may also be made by the incorporation of one or more species presenting an –OH group, preferably serine or threonine residues, within the sequence of the peptide (for O-linked glycosylation sites). The addition may be made by mutation or by full chemical synthesis of the peptide. The peptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the

peptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) are preferably made using methods known in the art.

In an exemplary embodiment, the glycosylation site is added by shuffling polynucleotides. Polynucleotides encoding a candidate peptide can be modulated with DNA shuffling protocols. DNA shuffling is a process of recursive recombination and mutation, performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. *See, e.g.*, Stemmer, *Proc. Natl. Acad. Sci. USA* 91:10747-10751 (1994); Stemmer, *Nature* 370:389-391 (1994); and U.S. Patent Nos. 5,605,793, 5,837,458, 5,830,721 and 5,811,238.

The present invention also provides means of adding (or removing) one or more selected glycosyl residues to a peptide, after which a modified sugar is conjugated to at least one selected glycosyl residue of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified sugar to a selected glycosyl residue that is either not present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified sugar to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation pattern of a glycopeptide is altered prior to the conjugation of the modified sugar by the removal of a carbohydrate residue from the glycopeptide. *See*, for example WO 98/31826.

Addition or removal of any carbohydrate moieties present on the glycopeptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the polypeptide variant to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin *et al., Arch. Biochem. Biophys.* **259**: 52 (1987) and by Edge *et al., Anal. Biochem.* **118**: 131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al., Meth. Enzymol.* **138**: 350 (1987).

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Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) consensus sites for N- and O-glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyltransferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulfhydryl groups such as those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC CRIT. REV. BIOCHEM., pp. 259-306 (1981).

In one embodiment, the invention provides a method for linking two or more peptides through a linking group. For example, the toxin can be a peptide. The linking group is of any useful structure and may be selected from straight-chain and branched chain structures. Preferably, each terminus of the linker, which is attached to a peptide, includes a modified sugar (i.e., a nascent intact glycosyl linking group).

The discussion that follows is also generally illustrative for embodiments in which the toxin is a molecule other than a peptide. In an exemplary method of the invention, two peptides are linked together via a linker moiety that includes a PEG linker. An exemplary construct of the invention includes two intact glycosyl linking groups. The focus on a PEG linker that includes two glycosyl groups is for purposes of clarity and should not be interpreted as limiting the identity of linker arms of use in this embodiment of the invention.

Thus, a PEG moiety is functionalized at a first terminus with a first glycosyl unit and at a second terminus with a second glycosyl unit. The first and second glycosyl units are preferably substrates for different transferases, allowing orthogonal attachment of the first and second peptides to the first and second glycosyl units, respectively. In practice, the $(glycosyl)^1$-PEG-$(glycosyl)^2$ linker is contacted with the first peptide and a first transferase for which the first glycosyl unit is a substrate, thereby forming $(peptide)^1$-$(glycosyl)^1$-PEG-$(glycosyl)^2$. Transferase and/or unreacted peptide is then optionally removed from the reaction mixture. The second peptide and a second transferase for which the second glycosyl unit is a substrate are added to the $(peptide)^1$-$(glycosyl)^1$-PEG-$(glycosyl)^2$ conjugate, forming $(peptide)^1$-$(glycosyl)^1$-PEG-$(glycosyl)^2$-$(peptide)^2$. Those of skill in the art will appreciate that the method outlined above is also applicable to forming conjugates between more than two peptides by, for example, the use of a branched PEG, dendrimer, poly(amino acid), polsaccharide or the like.

The processes described above can be carried through as many cycles as desired, and is not limited to forming a conjugate between two peptides with a single linker. Moreover, those of skill in the art will appreciate that the reactions functionalizing the intact glycosyl linking groups at the termini of the PEG (or other) linker with the peptide can occur simultaneously in the same reaction vessel, or they can be carried out in a step-wise fashion. When the reactions are carried out in a step-wise manner, the conjugate produced at each step is optionally purified from one or more reaction components (e.g., enzymes, peptides).

The use of reactive derivatives of PEG (or other linkers) to attach one or more peptide moieties to the linker is within the scope of the present invention. The invention is not limited by the identity of the reactive PEG analogue. Many activated derivatives of poly(ethylene glycol) are available commercially and preparations of these species are prevalent in the literature. It is well within the abilities of one of skill to choose, and synthesize if necessary, an appropriate activated PEG derivative with which to prepare a substrate useful in the present invention. *See*, Abuchowski *et al. Cancer Biochem. Biophys.*, 7: 175-186 (1984); Abuchowski *et al.*, *J. Biol. Chem.*, **252**: 3582-3586 (1977); Jackson *et al.*, *Anal. Biochem.*, **165**: 114-127 (1987); Koide *et al.*, *Biochem Biophys. Res. Commun.*, **111**: 659-667 (1983)), tresylate (Nilsson *et al.*, *Methods Enzymol.*, **104**: 56-69 (1984); Delgado *et al.*, *Biotechnol. Appl. Biochem.*, **12**: 119-128 (1990)); N-hydroxysuccinimide derived active esters (Buckmann *et al.*, *Makromol. Chem.*, **182**: 1379-1384 (1981); Joppich *et al.*, *Makromol. Chem.*, **180**: 1381-1384 (1979); Abuchowski *et al.*, *Cancer Biochem. Biophys.*, 7: 175-186 (1984); Katre*et al. Proc. Natl. Acad. Sci. U.S.A.*, **84**: 1487-1491 (1987); Kitamura *et al.*, *Cancer Res.*, **51**: 4310-4315 (1991); Boccu *et al.*, *Z. Naturforsch.*, **38C**: 94-99 (1983), carbonates (Zalipsky *et al.*, POLY(ETHYLENE GLYCOL) CHEMISTRY: BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, Harris, Ed., Plenum Press, New York, 1992, pp. 347-370; Zalipsky *et al.*, *Biotechnol. Appl. Biochem.*, **15**: 100-114 (1992); Veronese *et al.*, *Appl. Biochem. Biotech.*, **11**: 141-152 (1985)), imidazolyl formates (Beauchamp *et al.*, *Anal. Biochem.*, **131**: 25-33 (1983); Berger *et al.*, *Blood*, **71**: 1641-1647 (1988)), 4-dithiopyridines (Woghiren *et al.*, *Bioconjugate Chem.*, **4**: 314-318 (1993)), isocyanates (Byun *et al.*, *ASAIO Journal*, M649-M-653 (1992)) and epoxides (U.S. Pat. No. 4,806,595, issued to Noishiki *et al.*, (1989). Other linking groups include the urethane linkage between amino groups and activated PEG. *See*, Veronese, *et al.*, *Appl. Biochem. Biotechnol.*, **11**: 141-152 (1985).

Preparation of Modified Sugars

In general, the sugar moiety and the modifying group are linked together through the use of reactive groups, which are typically transformed by the linking process into a new organic functional group or inert species. The sugar reactive functional group(s), is located at any position on the sugar moiety. Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. Currently favored classes of reactions available with reactive sugar moieties are those, which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American Chemical Society, Washington, D.C., 1982.

Useful reactive functional groups pendent from a sugar nucleus, toxin, spacer, amplifier or other component of a conjugate of the invention include, but are not limited to:

(a) carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters;

(b) hydroxyl groups, which can be converted to, *e.g.*, esters, ethers, aldehydes, *etc.*

(c) haloalkyl groups, wherein the halide can be later displaced with a nucleophilic group such as, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion, thereby resulting in the covalent attachment of a new group at the functional group of the halogen atom;

(d) dienophile groups, which are capable of participating in Diels-Alder reactions such as, for example, maleimido groups;

(e) aldehyde or ketone groups, such that subsequent derivatization is possible via formation of carbonyl derivatives such as, for example, imines, hydrazones,

semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition;

(f) sulfonyl halide groups for subsequent reaction with amines, for example, to form sulfonamides;

(g) thiol groups, which can be, for example, converted to disulfides or reacted with acyl halides;

(h) amine or sulfhydryl groups, which can be, for example, acylated, alkylated or oxidized;

(i) alkenes, which can undergo, for example, cycloadditions, acylation, Michael addition, *etc*; and

(j) epoxides, which can react with, for example, amines and hydroxyl compounds.

The reactive functional groups can be chosen such that they do not participate in, or interfere with, the reactions necessary to assemble the reactive sugar nucleus or modifying group. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art understand how to protect a particular functional group such that it does not interfere with a chosen set of reaction conditions. For examples of useful protecting groups, *see,* for example, Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

In the discussion that follows, a number of specific examples of modified sugars that are useful in practicing the present invention are set forth. In the exemplary embodiments, a sialic acid derivative is utilized as the sugar nucleus to which the modifying group is attached. The focus of the discussion on sialic acid derivatives is for clarity of illustration only and should not be construed to limit the scope of the invention. Those of skill in the art will appreciate that a variety of other sugar moieties can be activated and derivatized in a manner analogous to that set forth using sialic acid as an example. For example, numerous methods are available for modifying galactose, glucose, N-acetylgalactosamine and fucose to name a few sugar substrates, which are readily modified by art recognized methods. *See*, for example, Elhalabi *et al.*, *Curr. Med. Chem.* **6**: 93 (1999); and Schafer *et al.*, *J. Org. Chem.* **65**: 24 (2000)).

In an exemplary embodiment, the peptide that is modified by a method of the invention is a glycopeptide that is produced in mammalian cells (e.g., CHO cells) or in a

transgenic animal and thus, contains N- and/or O-linked oligosaccharide chains, which are incompletely sialylated. The oligosaccharide chains of the glycopeptide lacking a sialic acid and containing a terminal galactose residue can be PEGylated, PPGylated or otherwise modified with a modified sialic acid.

In Scheme 1, the amino glycoside **1**, is treated with the active ester of a protected amino acid (*e.g.*, glycine) derivative, converting the sugar amine residue into the corresponding protected amino acid amide adduct. The adduct is treated with an aldolase to form α-hydroxy carboxylate **2**. Compound **2** is converted to the corresponding CMP derivative by the action of CMP-SA synthetase, followed by catalytic hydrogenation of the CMP derivative to produce compound **3**. The amine introduced via formation of the glycine adduct is utilized as a locus of toxin-PEG or PPG attachment by reacting compound **3** with an activated toxin-PEG or toxin-PPG derivative (*e.g.*, toxin-PEG-C(O)NHS, toxin-PPG-C(O)NHS), producing **4** or **5**, respectively.

Scheme 1

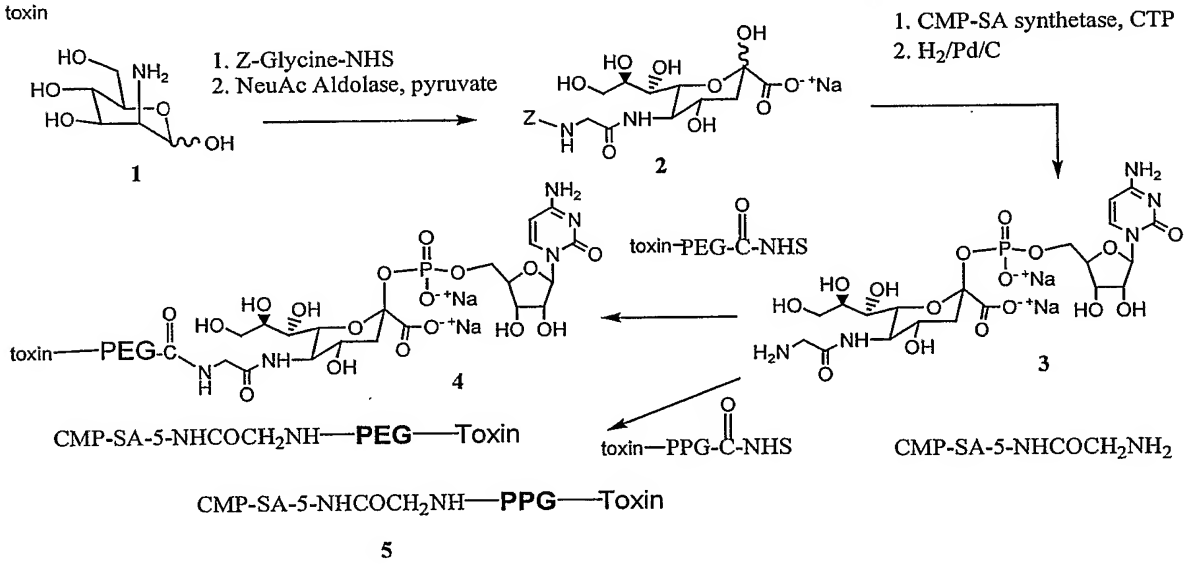
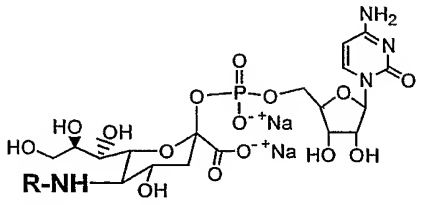


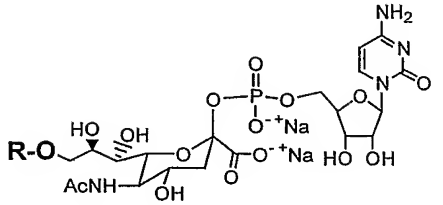
Table 1 sets forth representative examples of sugar monophosphates that are derivatized with a toxin-PEG or toxin-PPG moiety. Certain of the compounds of Table 1 are prepared by the method of Scheme 1. Other derivatives are prepared by art-recognized methods. *See*, for example, Keppler *et al.*, *Glycobiology* **11**: 11R (2001); and Charter *et al.*, *Glycobiology* **10**: 1049 (2000)). Other amine reactive PEG and PPG analogues are commercially available, or they can be prepared by methods readily accessible to those of

skill in the art. "R," in Table 1 represents a spacer, a spacer-toxin, or a spacer-toxin-amplifier construct as discussed above.

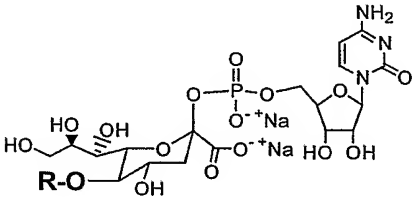
Table 1



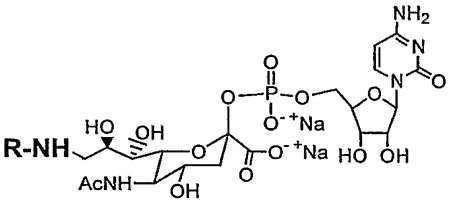
CMP-SA-5-NH-R



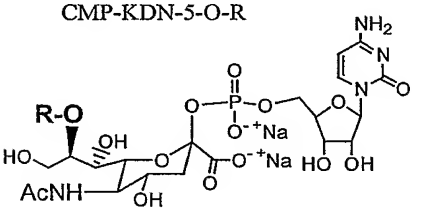
CMP-NeuAc-9-O-R



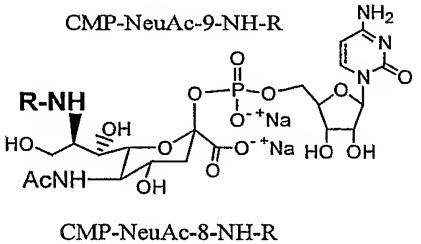
CMP-KDN-5-O-R



CMP-NeuAc-9-NH-R



CMP-NeuAc-8-O-R



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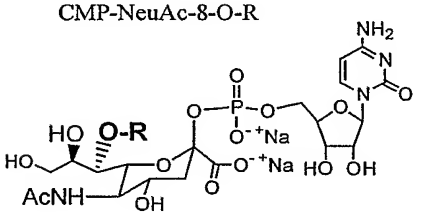

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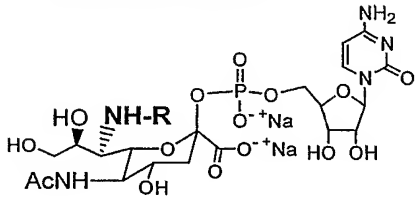
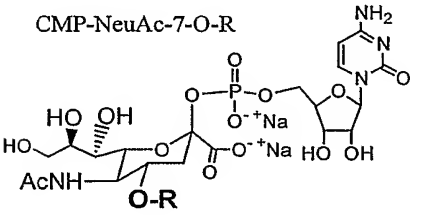
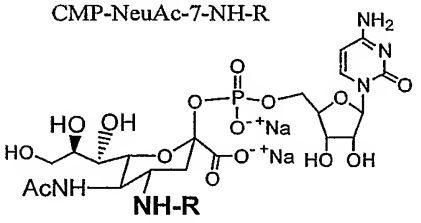


CMP-NeuAc-7-NH-R

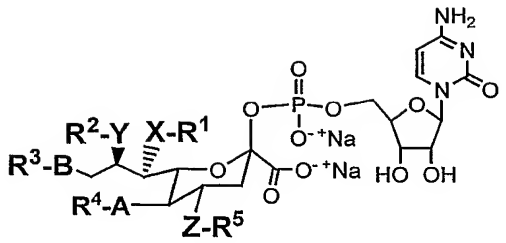


CMP-NeuAc-4-O-R



CMP-NeuAc-4-NH-R

The modified sugar phosphates of use in practicing the present invention can be substituted in other positions as well as those set forth above. Presently preferred substitutions of sialic acid are set forth in Formula I:



(I)

in which X, Y, Z, A and B are independently selected from linking groups, which are preferably selected from –O-, -N(R)-, -S, CR_2-, and $N(R)_2$, in which each R is a member independently selected from H, OH, substituted or unsubstituted alkyl, and substituted or unsubstituted heteroalkyl. The symbols R^1, R^2, R^3, R^4 and R^5 represent H, OH, a spacer or spacer-toxin construct as discussed above. Alternatively, R^1-R^5 represent a water-soluble polymer, therapeutic moiety, biomolecule or other moiety.

Exemplary moieties attached to the conjugates disclosed herein include, but are not limited to, PEG derivatives (*e.g.*, acyl-PEG, acyl-alkyl-PEG, alkyl-acyl-PEG carbamoyl-PEG, aryl-PEG), PPG derivatives (*e.g.*, acyl-PPG, acyl-alkyl-PPG, alkyl-acyl-PPG carbamoyl-PPG, aryl-PPG), therapeutic moieties, diagnostic moieties, mannose-6-phosphate, heparin, heparan, SLe_x, mannose, mannose-6-phosphate, Sialyl Lewis X, FGF, VFGF, proteins, chondroitin, keratan, dermatan, albumin, integrins, antennary oligosaccharides, peptides and the like. Methods of conjugating the various modifying groups to a saccharide moiety are readily accessible to those of skill in the art (POLY (ETHYLENE GLYCOL CHEMISTRY : BIOTECHNICAL AND BIOMEDICAL APPLICATIONS, J. Milton Harris, Ed., Plenum Pub. Corp., 1992; POLY (ETHYLENE GLYCOL) CHEMICAL AND BIOLOGICAL APPLICATIONS, J. Milton Harris, Ed., ACS Symposium Series No. 680, American Chemical Society, 1997; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991).

Water-Insoluble Polymers

In another embodiment, analogous to those discussed above, the modifying group is a water-insoluble polymer, rather than a water-soluble polymer. The glyco-conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic peptide in a controlled manner. Polymeric drug delivery systems are known in the art. *See*, for example, Dunn *et al.*, Eds. POLYMERIC DRUGS AND DRUG

DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991. Those of skill in the art will appreciate that substantially any known drug delivery system is applicable to the conjugates of the present invention.

Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes, polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly (ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronics and polyvinylphenol and copolymers thereof.

Synthetically modified natural polymers of use in the glyco-conjugates of the invention include, but are not limited to, alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, and nitrocelluloses. Particularly preferred members of the broad classes of synthetically modified natural polymers include, but are not limited to, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxymethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, and polymers of acrylic and methacrylic esters and alginic acid.

These and the other polymers discussed herein can be readily obtained from commercial sources such as Sigma Chemical Co. (St. Louis, MO.), Polysciences (Warrenton, PA.), Aldrich (Milwaukee, WI.), Fluka (Ronkonkoma, NY), and BioRad (Richmond, CA), or else synthesized from monomers obtained from these suppliers using standard techniques.

Cross-linking Groups

Preparation of the modified sugar for use in the methods of the present invention includes attachment of a modifying group to a sugar residue and forming a stable adduct, which is a substrate for an enzyme, *e.g.*, or a glycosyltransferase. Thus, a cross-linking agent is often used to conjugate the modifying group and the sugar. Exemplary

bifunctional compounds which can be used for attaching modifying groups to carbohydrate moieties include, but are not limited to, bifunctional poly(ethyleneglycols), polyamides, polyethers, polyesters and the like. General approaches for linking carbohydrates to other molecules are known in the literature. *See,* for example, Lee *et al.*, *Biochemistry* **28**: 1856 (1989); Bhatia *et al.*, *Anal. Biochem.* **178**: 408 (1989); Janda *et al.*, *J. Am. Chem. Soc.* **112**: 8886 (1990) and Bednarski *et al.*, WO 92/18135. In the discussion that follows, the reactive groups are treated as benign on the sugar moiety of the nascent modified sugar. The focus of the discussion is for clarity of illustration. Those of skill in the art will appreciate that the discussion is relevant to reactive groups on the modifying group as well.

An exemplary strategy involves incorporation of a protected sulfhydryl onto the sugar using the heterobifunctional crosslinker SPDP (n-succinimidyl-3-(2-pyridyldithio)propionate and then deprotecting the sulfhydryl for formation of a disulfide bond with another sulfhydryl on the modifying group.

If SPDP detrimentally affects the ability of the modified sugar to act as a glycosyltransferase substrate, one of an array of other crosslinkers such as 2-iminothiolane or N-succinimidyl S-acetylthioacetate (SATA) is used to form a disulfide bond. 2-iminothiolane reacts with primary amines, instantly incorporating an unprotected sulfhydryl onto the amine-containing molecule. SATA also reacts with primary amines, but incorporates a protected sulfhydryl, which is later deacetaylated using hydroxylamine to produce a free sulfhydryl. In each case, the incorporated sulfhydryl is free to react with other sulfhydryls or protected sulfhydryl, like SPDP, forming the required disulfide bond.

The above-described strategy is exemplary, and not limiting, of linkers of use in the invention. Other crosslinkers are available that can be used in different strategies for crosslinking the modifying group to the peptide. For example, TPCH(S-(2-thiopyridyl)-L-cysteine hydrazide and TPMPH ((S-(2-thiopyridyl) mercapto-propionohydrazide) react with carbohydrate moieties that have been previously oxidized by mild periodate treatment, thus forming a hydrazone bond between the hydrazide portion of the crosslinker and the periodate generated aldehydes. TPCH and TPMPH introduce a 2-pyridylthione protected sulfhydryl group onto the sugar, which can be deprotected with DTT and then subsequently used for conjugation, such as forming disulfide bonds between components.

If disulfide bonding is found unsuitable for producing stable modified sugars, other crosslinkers may be used that incorporate more stable bonds between components. The heterobifunctional crosslinkers GMBS (N-gama-malimidobutyryloxy)succinimide) and SMCC (succinimidyl 4-(N-maleimido-methyl)cyclohexane) react with primary amines, thus

introducing a maleimide group onto the component. The maleimide group can subsequently react with sulfhydryls on the other component, which can be introduced by previously mentioned crosslinkers, thus forming a stable thioether bond between the components. If steric hindrance between components interferes with either component's activity or the ability of the modified sugar to act as a glycosyltransferase substrate, crosslinkers can be used which introduce long spacer arms between components and include derivatives of some of the previously mentioned crosslinkers (*i.e.*, SPDP). Thus, there is an abundance of suitable crosslinkers, which are useful; each of which is selected depending on the effects it has on optimal peptide conjugate and modified sugar production.

A variety of reagents are used to modify the components of the modified sugar with intramolecular chemical crosslinks (for reviews of crosslinking reagents and crosslinking procedures see: Wold, F., *Meth. Enzymol.* **25**: 623-651, 1972; Weetall, H. H., and Cooney, D. A., In: ENZYMES AS DRUGS. (Holcenberg, and Roberts, eds.) pp. 395-442, Wiley, New York, 1981; Ji, T. H., *Meth. Enzymol.* **91**: 580-609, 1983; Mattson *et al.*, *Mol. Biol. Rep.* **17**: 167-183, 1993, all of which are incorporated herein by reference). Preferred crosslinking reagents are derived from various zero-length, homo-bifunctional, and hetero-bifunctional crosslinking reagents. Zero-length crosslinking reagents include direct conjugation of two intrinsic chemical groups with no introduction of extrinsic material. Agents that catalyze formation of a disulfide bond belong to this category. Another example is reagents that induce condensation of a carboxyl and a primary amino group to form an amide bond such as carbodiimides, ethylchloroformate, Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate), and carbonyldiimidazole. In addition to these chemical reagents, the enzyme transglutaminase (glutamyl-peptide γ-glutamyltransferase; EC 2.3.2.13) may be used as zero-length crosslinking reagent. This enzyme catalyzes acyl transfer reactions at carboxamide groups of protein-bound glutaminyl residues, usually with a primary amino group as substrate. Preferred homo- and hetero-bifunctional reagents contain two identical or two dissimilar sites, respectively, which may be reactive for amino, sulfhydryl, guanidino, indole, or nonspecific groups.

i. Preferred Specific Sites in Crosslinking Reagents

1. <u>Amino-Reactive Groups</u>

In one preferred embodiment, the sites on the cross-linker are amino-reactive groups. Useful non-limiting examples of amino-reactive groups include N-

hydroxysuccinimide (NHS) esters, imidoesters, isocyanates, acylhalides, arylazides, p-nitrophenyl esters, aldehydes, and sulfonyl chlorides.

NHS esters react preferentially with the primary (including aromatic) amino groups of a modified sugar component. The imidazole groups of histidines are known to compete with primary amines for reaction, but the reaction products are unstable and readily hydrolyzed. The reaction involves the nucleophilic attack of an amine on the acid carboxyl of an NHS ester to form an amide, releasing the N-hydroxysuccinimide. Thus, the positive charge of the original amino group is lost.

Imidoesters are the most specific acylating reagents for reaction with the amine groups of the modified sugar components. At a pH between 7 and 10, imidoesters react only with primary amines. Primary amines attack imidates nucleophilically to produce an intermediate that breaks down to amidine at high pH or to a new imidate at low pH. The new imidate can react with another primary amine, thus crosslinking two amino groups, a case of a putatively monofunctional imidate reacting bifunctionally. The principal product of reaction with primary amines is an amidine that is a stronger base than the original amine. The positive charge of the original amino group is therefore retained.

Isocyanates (and isothiocyanates) react with the primary amines of the modified sugar components to form stable bonds. Their reactions with sulfhydryl, imidazole, and tyrosyl groups give relatively unstable products.

Acylazides are also used as amino-specific reagents in which nucleophilic amines of the affinity component attack acidic carboxyl groups under slightly alkaline conditions, *e.g.* pH 8.5.

Arylhalides such as 1,5-difluoro-2,4-dinitrobenzene react preferentially with the amino groups and tyrosine phenolic groups of modified sugar components, but also with sulfhydryl and imidazole groups.

p-Nitrophenyl esters of mono- and dicarboxylic acids are also useful amino-reactive groups. Although the reagent specificity is not very high, α- and ε-amino groups appear to react most rapidly.

Aldehydes such as glutaraldehyde react with primary amines of modified sugar. Although unstable Schiff bases are formed upon reaction of the amino groups with the aldehydes of the aldehydes, glutaraldehyde is capable of modifying the modified sugar with stable crosslinks. At pH 6-8, the pH of typical crosslinking conditions, the cyclic polymers undergo a dehydration to form α-β unsaturated aldehyde polymers. Schiff bases, however,

are stable, when conjugated to another double bond. The resonant interaction of both double bonds prevents hydrolysis of the Schiff linkage. Furthermore, amines at high local concentrations can attack the ethylenic double bond to form a stable Michael addition product.

Aromatic sulfonyl chlorides react with a variety of sites of the modified sugar components, but reaction with the amino groups is the most important, resulting in a stable sulfonamide linkage.

2. Sulfhydryl-Reactive Groups

In another preferred embodiment, the sites are sulfhydryl-reactive groups. Useful, non-limiting examples of sulfhydryl-reactive groups include maleimides, alkyl halides, pyridyl disulfides, and thiophthalimides.

Maleimides react preferentially with the sulfhydryl group of the modified sugar components to form stable thioether bonds. They also react at a much slower rate with primary amino groups and the imidazole groups of histidines. However, at pH 7 the maleimide group can be considered a sulfhydryl-specific group, since at this pH the reaction rate of simple thiols is 1000-fold greater than that of the corresponding amine.

Alkyl halides react with sulfhydryl groups, sulfides, imidazoles, and amino groups. At neutral to slightly alkaline pH, however, alkyl halides react primarily with sulfhydryl groups to form stable thioether bonds. At higher pH, reaction with amino groups is favored.

Pyridyl disulfides react with free sulfhydryls via disulfide exchange to give mixed disulfides. As a result, pyridyl disulfides are the most specific sulfhydryl-reactive groups.

Thiophthalimides react with free sulfhydryl groups to form disulfides.

3. Carboxyl-Reactive Residue

In another embodiment, carbodiimides soluble in both water and organic solvent, are used as carboxyl-reactive reagents. These compounds react with free carboxyl groups forming a pseudourea that can then couple to available amines yielding an amide linkage teach how to modify a carboxyl group with carbodiimde (Yamada *et al.*, *Biochemistry* **20**: 4836-4842, 1981).

ii. Preferred Nonspecific Sites in Crosslinking Reagents

In addition to the use of site-specific reactive moieties, the present invention contemplates the use of non-specific reactive groups to link the sugar to the modifying group.

Exemplary non-specific cross-linkers include photoactivatable groups, completely inert in the dark, which are converted to reactive species upon absorption of a photon of appropriate energy. In one preferred embodiment, photoactivatable groups are selected from precursors of nitrenes generated upon heating or photolysis of azides. Electron-deficient nitrenes are extremely reactive and can react with a variety of chemical bonds including N-H, O-H, C-H, and C=C. Although three types of azides (aryl, alkyl, and acyl derivatives) may be employed, arylazides are presently preferred. The reactivity of arylazides upon photolysis is better with N-H and O-H than C-H bonds. Electron-deficient arylnitrenes rapidly ring-expand to form dehydroazepines, which tend to react with nucleophiles, rather than form C-H insertion products. The reactivity of arylazides can be increased by the presence of electron-withdrawing substituents such as nitro or hydroxyl groups in the ring. Such substituents push the absorption maximum of arylazides to longer wavelength. Unsubstituted arylazides have an absorption maximum in the range of 260-280 nm, while hydroxy and nitroarylazides absorb significant light beyond 305 nm. Therefore, hydroxy and nitroarylazides are most preferable since they allow to employ less harmful photolysis conditions for the affinity component than unsubstituted arylazides.

In another preferred embodiment, photoactivatable groups are selected from fluorinated arylazides. The photolysis products of fluorinated arylazides are arylnitrenes, all of which undergo the characteristic reactions of this group, including C-H bond insertion, with high efficiency (Keana *et al., J. Org. Chem.* **55**: 3640-3647, 1990).

In another embodiment, photoactivatable groups are selected from benzophenone residues. Benzophenone reagents generally give higher crosslinking yields than arylazide reagents.

In another embodiment, photoactivatable groups are selected from diazo compounds, which form an electron-deficient carbene upon photolysis. These carbenes undergo a variety of reactions including insertion into C-H bonds, addition to double bonds (including aromatic systems), hydrogen attraction and coordination to nucleophilic centers to give carbon ions.

In still another embodiment, photoactivatable groups are selected from diazopyruvates. For example, the p-nitrophenyl ester of p-nitrophenyl diazopyruvate reacts with aliphatic amines to give diazopyruvic acid amides that undergo ultraviolet photolysis to

form aldehydes. The photolyzed diazopyruvate-modified affinity component will react like formaldehyde or glutaraldehyde forming crosslinks.

iii. Homobifunctional Reagents

1. Homobifunctional crosslinkers reactive with primary amines

Synthesis, properties, and applications of amine-reactive cross-linkers are commercially described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many reagents are available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR.).

Preferred, non-limiting examples of homobifunctional NHS esters include disuccinimidyl glutarate (DSG), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS), disuccinimidyl tartarate (DST), disulfosuccinimidyl tartarate (sulfo-DST), bis-2-(succinimidooxycarbonyloxy)ethylsulfone (BSOCOES), bis-2-(sulfosuccinimidooxy-carbonyloxy)ethylsulfone (sulfo-BSOCOES), ethylene glycolbis(succinimidylsuccinate) (EGS), ethylene glycolbis(sulfosuccinimidylsuccinate) (sulfo-EGS), dithiobis(succinimidyl-propionate (DSP), and dithiobis(sulfosuccinimidylpropionate (sulfo-DSP). Preferred, non-limiting examples of homobifunctional imidoesters include dimethyl malonimidate (DMM), dimethyl succinimidate (DMSC), dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP), dimethyl suberimidate (DMS), dimethyl-3,3'-oxydipropionimidate (DODP), dimethyl-3,3'-(methylenedioxy)dipropionimidate (DMDP), dimethyl-,3'-(dimethylenedioxy)dipropionimidate (DDDP), dimethyl-3,3'-(tetramethylenedioxy)-dipropionimidate (DTDP), and dimethyl-3,3'-dithiobispropionimidate (DTBP).

Preferred, non-limiting examples of homobifunctional isothiocyanates include: p-phenylenediisothiocyanate (DITC), and 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS).

Preferred, non-limiting examples of homobifunctional isocyanates include xylene-diisocyanate, toluene-2,4-diisocyanate, toluene-2-isocyanate-4-isothiocyanate, 3-methoxydiphenylmethane-4,4'-diisocyanate, 2,2'-dicarboxy-4,4'-azophenyldiisocyanate, and hexamethylenediisocyanate.

Preferred, non-limiting examples of homobifunctional arylhalides include 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and 4,4'-difluoro-3,3'-dinitrophenyl-sulfone.

Preferred, non-limiting examples of homobifunctional aliphatic aldehyde reagents include glyoxal, malondialdehyde, and glutaraldehyde.

Preferred, non-limiting examples of homobifunctional acylating reagents include nitrophenyl esters of dicarboxylic acids.

Preferred, non-limiting examples of homobifunctional aromatic sulfonyl chlorides include phenol-2,4-disulfonyl chloride, and α-naphthol-2,4-disulfonyl chloride.

Preferred, non-limiting examples of additional amino-reactive homobifunctional reagents include erythritolbiscarbonate which reacts with amines to give biscarbamates.

2. Homobifunctional Crosslinkers Reactive with Free Sulfhydryl Groups

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of homobifunctional maleimides include bismaleimidohexane (BMH), N,N'-(1,3-phenylene) bismaleimide, N,N'-(1,2-phenylene)bismaleimide, azophenyldimaleimide, and bis(N-maleimidomethyl)ether.

Preferred, non-limiting examples of homobifunctional pyridyl disulfides include 1,4-di-3'-(2'-pyridyldithio)propionamidobutane (DPDPB).

Preferred, non-limiting examples of homobifunctional alkyl halides include 2,2'-dicarboxy-4,4'-diiodoacetamidoazobenzene, α,α'-diiodo-p-xylenesulfonic acid, α, α'-dibromo-p-xylenesulfonic acid, N,N'-bis(b-bromoethyl)benzylamine, N,N'-di(bromoacetyl)phenylthydrazine, and 1,2-di(bromoacetyl)amino-3-phenylpropane.

3. Homobifunctional Photoactivatable Crosslinkers

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Some of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of homobifunctional photoactivatable crosslinker include bis-β-(4-azidosalicylamido)ethyldisulfide (BASED), di-N-(2-nitro-4-azidophenyl)-cystamine-S,S-dioxide (DNCO), and 4,4'-dithiobisphenylazide.

iv. HeteroBifunctional Reagents

1. Amino-Reactive HeteroBifunctional Reagents with a Pyridyl Disulfide Moiety

Synthesis, properties, and applications of such reagents are described in the literature (for reviews of crosslinking procedures and reagents, *see above*). Many of the reagents are commercially available (*e.g.*, Pierce Chemical Company, Rockford, Ill.; Sigma Chemical Company, St. Louis, Mo.; Molecular Probes, Inc., Eugene, OR).

Preferred, non-limiting examples of hetero-bifunctional reagents with a pyridyl disulfide moiety and an amino-reactive NHS ester include N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), succinimidyl 6-3-(2-pyridyldithio)propionamidohexanoate (LC-SPDP), sulfosuccinimidyl 6-3-(2-pyridyldithio)propionamidohexanoate (sulfo-LCSPDP), 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT), and sulfosuccinimidyl 6-α-methyl-α-(2-pyridyldithio)toluamidohexanoate (sulfo-LC-SMPT).

2. Amino-Reactive HeteroBifunctional Reagents with a Maleimide Moiety

Synthesis, properties, and applications of such reagents are described in the literature. Preferred, non-limiting examples of hetero-bifunctional reagents with a maleimide moiety and an amino-reactive NHS ester include succinimidyl maleimidylacetate (AMAS), succinimidyl 3-maleimidylpropionate (BMPS), N- γ-maleimidobutyryloxysuccinimide ester (GMBS)N-γ-maleimidobutyryloxysulfo succinimide ester (sulfo-GMBS) succinimidyl 6-maleimidylhexanoate (EMCS), succinimidyl 3-maleimidylbenzoate (SMB), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS), succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), and sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate (sulfo-SMPB).

3. Amino-Reactive HeteroBifunctional Reagents with an Alkyl Halide Moiety

Synthesis, properties, and applications of such reagents are described in the literature Preferred, non-limiting examples of hetero-bifunctional reagents with an alkyl halide moiety and an amino-reactive NHS ester include N-succinimidyl-(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl-(4-iodoacetyl)aminobenzoate (sulfo-SIAB), succinimidyl-6-(iodoacetyl)aminohexanoate (SIAX), succinimidyl-6-(6-((iodoacetyl)-

amino)hexanoylamino)hexanoate (SIAXX), succinimidyl-6-(((4-(iodoacetyl)-amino)-methyl)-cyclohexane-1-carbonyl)aminohexanoate (SIACX), and succinimidyl-4((iodoacetyl)-amino)methylcyclohexane-1-carboxylate (SIAC).

A preferred example of a hetero-bifunctional reagent with an amino-reactive NHS ester and an alkyl dihalide moiety is N-hydroxysuccinimidyl 2,3-dibromopropionate (SDBP). SDBP introduces intramolecular crosslinks to the affinity component by conjugating its amino groups. The reactivity of the dibromopropionyl moiety towards primary amine groups is controlled by the reaction temperature (McKenzie *et al., Protein Chem.* 7: 581-592 (1988)).

Preferred, non-limiting examples of hetero-bifunctional reagents with an alkyl halide moiety and an amino-reactive p-nitrophenyl ester moiety include p-nitrophenyl iodoacetate (NPIA).

Other cross-linking agents are known to those of skill in the art. *See*, for example, Pomato *et al.*, U.S. Patent No. 5,965,106. It is within the abilities of one of skill in the art to choose an appropriate cross-linking agent for a particular application.

v. Cleavable Linker Groups

In yet a further embodiment, the linker group is provided with a group that can be cleaved to release the modifying group from the sugar residue. Many cleaveable groups are known in the art. *See,* for example, Jung *et al., Biochem. Biophys. Acta* **761**: 152-162 (1983); Joshi *et al., J. Biol. Chem.* **265**: 14518-14525 (1990); Zarling *et al., J. Immunol.* **124**: 913-920 (1980); Bouizar *et al., Eur. J. Biochem.* **155**: 141-147 (1986); Park *et al., J. Biol. Chem.* **261**: 205-210 (1986); Browning *et al., J. Immunol.* **143**: 1859-1867 (1989). Moreover a broad range of cleavable, bifunctional (both homo- and hetero-bifunctional) linker groups is commercially available from suppliers such as Pierce.

Exemplary cleaveable moieties can be cleaved using light, heat or reagents such as thiols, hydroxylamine, bases, periodate and the like. Moreover, certain preferred groups are cleaved *in vivo* in response to being endocytized (*e.g.*, cis-aconityl; *see,* Shen *et al., Biochem. Biophys. Res. Commun.* **102**: 1048 (1991)). Preferred cleaveable groups comprise a cleaveable moiety which is a member selected from the group consisting of disulfide, ester, imide, carbonate, nitrobenzyl, phenacyl and benzoin groups.

Conjugation of Modified Sugars to Peptides

The modified sugars are conjugated to a glycosylated or non-glycosylated peptide using an appropriate enzyme to mediate the conjugation. Preferably, the concentrations of the modified donor sugar(s), enzyme(s) and acceptor peptide(s) are selected such that glycosylation proceeds until the acceptor is consumed. The considerations discussed below, while set forth in the context of a sialyltransferase, are generally applicable to other glycosyltransferase reactions.

The present invention is practiced using a single glycosyltransferase or a combination of glycosyltransferases. For example, one can use a combination of a sialyltransferase and a galactosyltransferase. In those embodiments using more than one enzyme, the enzymes and substrates are preferably combined in an initial reaction mixture, or the enzymes and reagents for a second enzymatic reaction are added to the reaction medium once the first enzymatic reaction is complete or nearly complete. By conducting two enzymatic reactions in sequence in a single vessel, overall yields are improved over procedures in which an intermediate species is isolated. Moreover, cleanup and disposal of extra solvents and by-products is reduced.

In a preferred embodiment, each of the first and second enzyme is a glycosyltransferase. In another preferred embodiment, one enzyme is an endoglycosidase. In an additional preferred embodiment, more than two enzymes are used to assemble the modified glycoprotein of the invention. The enzymes are used to alter a saccharide structure on the peptide at any point either before or after the addition of the modified sugar to the peptide.

In another embodiment, the method makes use of one or more exo- or endoglycosidase. The glycosidase is typically a mutant, which is engineered to form glycosyl bonds rather than rupture them. The mutant glycanase typically includes a substitution of an amino acid residue for an active site acidic amino acid residue. For example, when the endoglycanase is endo-H, the substituted active site residues will typically be Asp at position 130, Glu at position 132 or a combination thereof. The amino acids are generally replaced with serine, alanine, asparagine, or glutamine (*see e.g.* U.S. Patent Application Publication 2004/0142856, serial number 410,913, filed April 9, 2003; U.S. Patent Application Publication 2004/0137557, serial number 287,994, filed November 5, 2003; and WO 03/031464 A2, published April 17, 2003).

The mutant enzyme catalyzes the reaction, usually by a synthesis step that is analogous to the reverse reaction of the endoglycanase hydrolysis step. In these

embodiments, the glycosyl donor molecule (*e.g.*, a desired oligo- or mono-saccharide structure) contains a leaving group and the reaction proceeds with the addition of the donor molecule to a GlcNAc residue on the protein. For example, the leaving group can be a halogen, such as fluoride. In other embodiments, the leaving group is a Asn, or a Asn-peptide moiety. In yet further embodiments, the GlcNAc residue on the glycosyl donor molecule is modified. For example, the GlcNAc residue may comprise a 1,2 oxazoline moiety.

In a preferred embodiment, each of the enzymes utilized to produce a conjugate of the invention is present in a catalytic amount. The catalytic amount of a particular enzyme varies according to the concentration of that enzyme's substrate as well as to reaction conditions such as temperature, time and pH value. Means for determining the catalytic amount for a given enzyme under preselected substrate concentrations and reaction conditions are well known to those of skill in the art.

The temperature at which an above process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denatures. Preferred temperature ranges are about 0 °C to about 55 °C, and more preferably about 20 ° C to about 30 °C. In another exemplary embodiment, one or more components of the present method are conducted at an elevated temperature using a thermophilic enzyme.

The reaction mixture is maintained for a period of time sufficient for the acceptor to be glycosylated, thereby forming the desired conjugate. Some of the conjugate can often be detected after a few hours, with recoverable amounts usually being obtained within 24 hours or less. Those of skill in the art understand that the rate of reaction is dependent on a number of variable factors (*e.g*, enzyme concentration, donor concentration, acceptor concentration, temperature, solvent volume), which are optimized for a selected system.

The present invention also provides for the industrial-scale production of modified peptides. As used herein, an industrial scale generally produces at least one gram of finsihed, purified conjugate.

In the discussion that follows, the invention is exemplified by the conjugation of modified sialic acid moieties to a glycosylated peptide. The exemplary modified sialic acid is labeled with PEG. The focus of the following discussion on the use of PEG-modified sialic acid and glycosylated peptides is for clarity of illustration and is not intended to imply that the invention is limited to the conjugation of these two partners. One of skill understands that the discussion is generally applicable to the additions of modified glycosyl moieties other

than sialic acid. Moreover, the discussion is equally applicable to the modification of a glycosyl unit with agents other than PEG including other water-soluble polymers, therapeutic moieties, and biomolecules.

An enzymatic approach can be used for the selective introduction of PEGylated or PPGylated carbohydrates onto a peptide or glycopeptide. The method utilizes modified sugars containing PEG, PPG, or a masked reactive functional group, and is combined with the appropriate glycosyltransferase or glycosynthase. By selecting the glycosyltransferase that will make the desired carbohydrate linkage and utilizing the modified sugar as the donor substrate, the PEG or PPG can be introduced directly onto the peptide backbone, onto existing sugar residues of a glycopeptide or onto sugar residues that have been added to a peptide.

An acceptor for the transferase, *e.g.* sialyltransferase, is present on the peptide to be modified by the methods of the present invention either as a naturally occurring structure or one placed there recombinantly, enzymatically or chemically. Suitable acceptors, include, for example, galactosyl acceptors such as Galβ1,4GlcNAc, Galβ1,4GalNAc, Galβ1,3GalNAc, lacto-N-tetraose, Galβ1,3GlcNAc, Galβ1,3Ara, Galβ1,6GlcNAc, Galβ1,4Glc (lactose), and other acceptors known to those of skill in the art (*see, e.g.*, Paulson *et al., J. Biol. Chem.* **253**: 5617-5624 (1978)).

In one embodiment, an acceptor for the sialyltransferase is present on the glycopeptide to be modified upon *in vivo* synthesis of the glycopeptide. Such glycopeptides can be sialylated using the claimed methods without prior modification of the glycosylation pattern of the glycopeptide. Alternatively, the methods of the invention can be used to sialylate a peptide that does not include a suitable acceptor; one first modifies the peptide to include an acceptor by methods known to those of skill in the art. In an exemplary embodiment, a GalNAc residue is added by the action of a GalNAc transferase.

In an exemplary embodiment, the galactosyl acceptor is assembled by attaching a galactose residue to an appropriate acceptor linked to the peptide, e.g., a GlcNAc. The method includes incubating the peptide to be modified with a reaction mixture that contains a suitable amount of a galactosyltransferase (*e.g.*, Galβ1,3 or Galβ1,4), and a suitable galactosyl donor (*e.g.*, UDP-galactose). The reaction is allowed to proceed substantially to completion or, alternatively, the reaction is terminated when a preselected amount of the galactose residue is added. Other methods of assembling a selected saccharide acceptor will be apparent to those of skill in the art.

In yet another embodiment, glycopeptide-linked oligosaccharides are first "trimmed," either in whole or in part, to expose either an acceptor for the sialyltransferase or a moiety to which one or more appropriate residues can be added to obtain a suitable acceptor. Enzymes such as glycosyltransferases and endoglycosidases (*see*, for example U.S. Patent No. 5,716,812) are useful for the attaching and trimming reactions.

In the discussion that follows, the method of the invention is exemplified by the use of modified sugars having a water-soluble polymer attached thereto. The focus of the discussion is for clarity of illustration. Those of skill will appreciate that the discussion is equally relevant to those embodiments in which the modified sugar bears a therapeutic moiety, biomolecule or the like.

An exemplary embodiment of the invention in which a carbohydrate residue is "trimmed" prior to the addition of the modified sugar is set forth in **FIG. 1**, which sets forth a scheme in which high mannose is trimmed back to the first generation biantennary structure. A modified sugar bearing a water-soluble polymer is conjugated to one or more of the sugar residues exposed by the "trimming back." In one example, a water-soluble polymer is added via a GlcNAc moiety conjugated to the water-soluble polymer. The modified GlcNAc is attached to one or both of the terminal mannose residues of the biantennary structure. Alternatively, an unmodified GlcNAc can be added to one or both of the termini of the branched species.

In another exemplary embodiment, a water-soluble polymer is added to one or both of the terminal mannose residues of the biantennary structure via a modified sugar having a galactose residue, which is conjugated to a GlcNAc residue added onto the terminal mannose residues. Alternatively, an unmodified Gal can be added to one or both terminal GlcNAc residues.

In yet a further example, a water-soluble polymer is added onto a Gal residue using a modified sialic acid.

Another exemplary embodiment is set forth in **FIG. 2**, which displays a scheme similar to that shown in **FIG. 1**, in which the high mannose structure is "trimmed back" to the mannose from which the biantennary structure branches. In one example, a water-soluble polymer is added via a GlcNAc modified with the polymer. Alternatively, an unmodified GlcNAc is added to the mannose, followed by a Gal with an attached water-soluble polymer. In yet another embodiment, unmodified GlcNAc and Gal residues are sequentially added to the mannose, followed by a sialic acid moiety modified with a water-soluble polymer.

FIG. 3 sets forth a further exemplary embodiment using a scheme similar to that shown in **FIG. 1**, in which high mannose is "trimmed back" to the GlcNAc to which the first mannose is attached. The GlcNAc is conjugated to a Gal residue bearing a water-soluble polymer. Alternatively, an unmodified Gal is added to the GlcNAc, followed by the addition of a sialic acid modified with a water-soluble sugar. In yet a further example, the terminal GlcNAc is conjugated with Gal and the GlcNAc is subsequently fucosylated with a modified fucose bearing a water-soluble polymer.

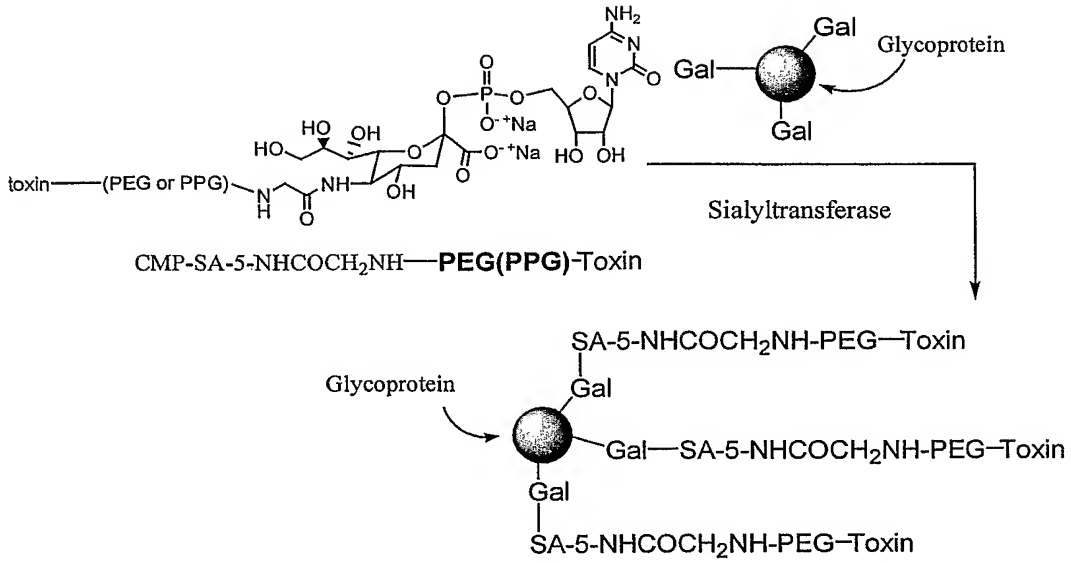
FIG. 4 is a scheme similar to that shown in **FIG. 1**, in which high mannose is trimmed back to the first GlcNAc attached to the Asn of the peptide. In one example, the GlcNAc of the GlcNAc-$(Fuc)_a$ residue is conjugated wit ha GlcNAc bearing a water soluble polymer. In another example, the GlcNAc of the GlcNAc-$(Fuc)_a$ residue is modified with Gal, which bears a water soluble polymer. In a still further embodiment, the GlcNAc is modified with Gal, followed by conjugation to the Gal of a sialic acid modified with a water-soluble polymer.

Other exemplary embodiments are set forth in **FIG. 5-9**. An illustration of the array of reaction types with which the present invention may be practiced is provided in **FIG. 10**.

The examples set forth above provide an illustration of the power of the methods set forth herein. Using the methods of the invention, it is possible to "trim back" and build up a carbohydrate residue of substantially any desired structure. The modified sugar can be added to the termini of the carbohydrate moiety as set forth above, or it can be intermediate between the peptide core and the terminus of the carbohydrate.

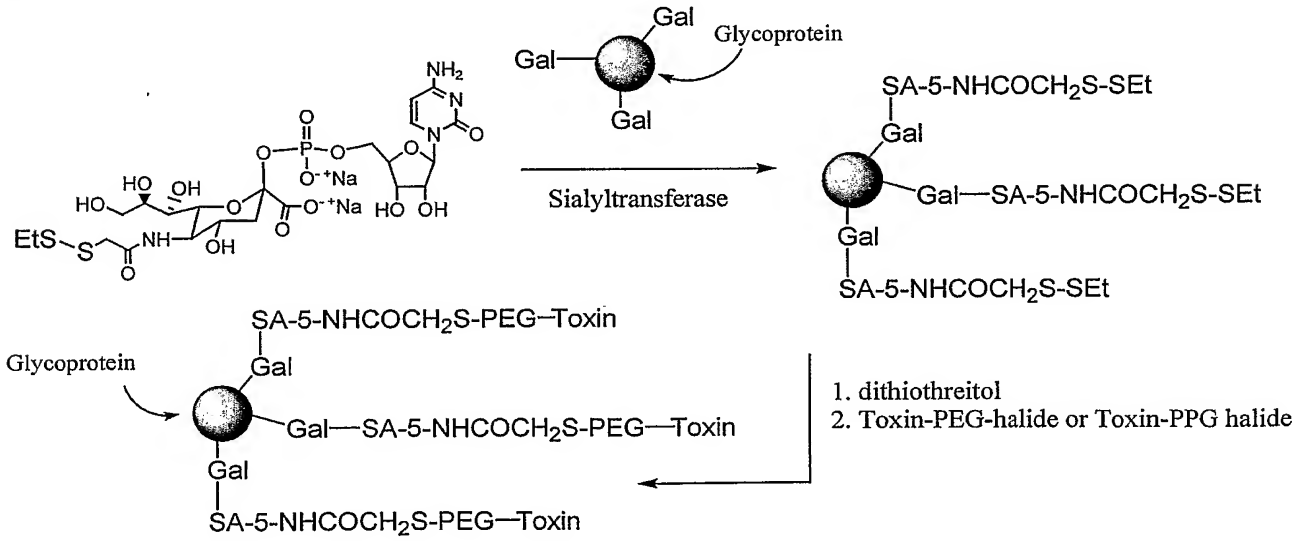
In an exemplary embodiment, an existing sialic acid is removed from a glycopeptide using a sialidase, thereby unmasking all or most of the underlying galactosyl residues. Alternatively, a peptide or glycopeptide is labeled with galactose residues, or an oligosaccharide residue that terminates in a galactose unit. Following the exposure of or addition of the galactose residues, an appropriate sialyltransferase is used to add a modified sialic acid. The approach is summarized in Scheme 2.

Scheme 2



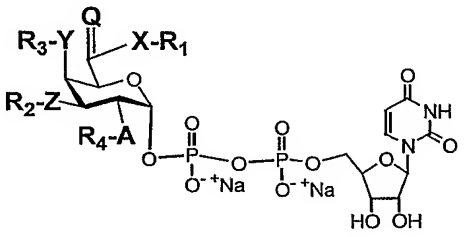
In yet a further approach, summarized in Scheme 3, a masked reactive functionality is present on the sialic acid. The masked reactive group is preferably unaffected by the conditions used to attach the modified sialic acid to the peptide. After the covalent attachment of the modified sialic acid to the peptide, the mask is removed and the peptide is conjugated with an agent such as PEG, PPG, a therapeutic moiety, biomolecule or other agent. The agent is conjugated to the peptide in a specific manner by its reaction with the unmasked reactive group on the modified sugar residue.

Scheme 3

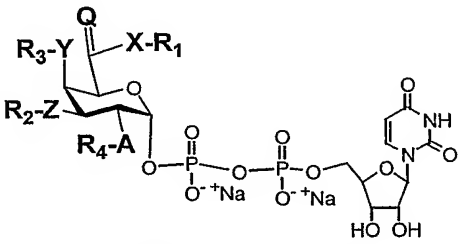


Any modified sugar can be used with its appropriate glycosyltransferase, depending on the terminal sugars of the oligosaccharide side chains of the glycopeptide (Table 2). As discussed above, the terminal sugar of the glycopeptide required for introduction of the PEGylated or PPGylated structure can be introduced naturally during expression or it can be produced post expression using the appropriate glycosidase(s), glycosyltransferase(s) or mix of glycosidase(s) and glycosyltransferase(s).

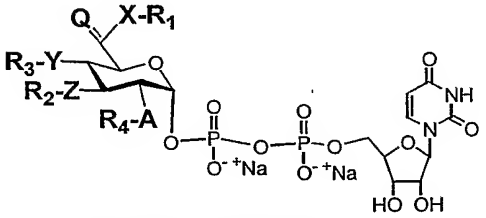
Table 2



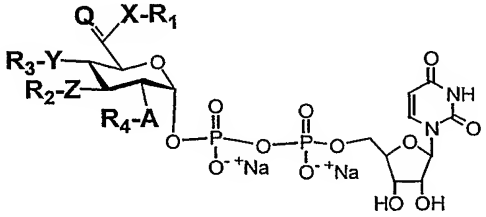
UDP-galactose-derivatives



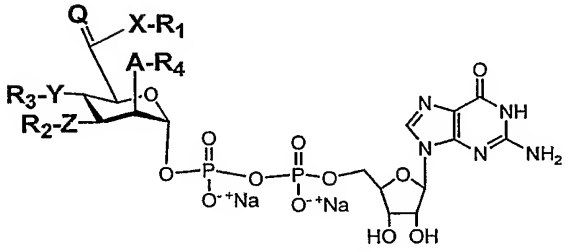
UDP-galactosamine-derivatives
(when A = NH, R_4 may be acetyl)



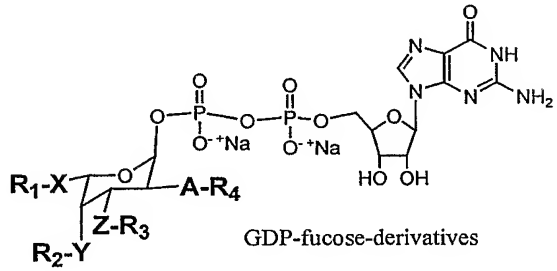
UDP-Glucose-derivatives



UDP-Glucosamine-derivatives
(when A = NH, R_4 may be acetyl)



GDP-Mannose-derivatives



GDP-fucose-derivatives

X = O, NH, S, CH_2, N-$(R_{1-5})_2$.
Y = X; Z = X; A = X; B = X.

Q = H_2, O, S, NH, N-R.

R, R_{1-4} = H, Spacer-M, M.

M = Ligand of interest

Ligand of interest = toxin, toxin-spacer, toxin-amplifier-spacer, acyl-PEG, acyl-PPG, alkyl-PEG, acyl-alkyl-PEG, acyl-alkyl-PEG, carbamoyl-PEG, carbamoyl-PPG, PEG, PPG, acyl-aryl-PEG, acyl-aryl-PPG, aryl-PEG, aryl-PPG, Mannose^{-6}-phosphate, heparin, heparan, SLex, Mannose, FGF, VFGF, protein, chondroitin, keratan, dermatan, albumin, integrins, peptides,etc.

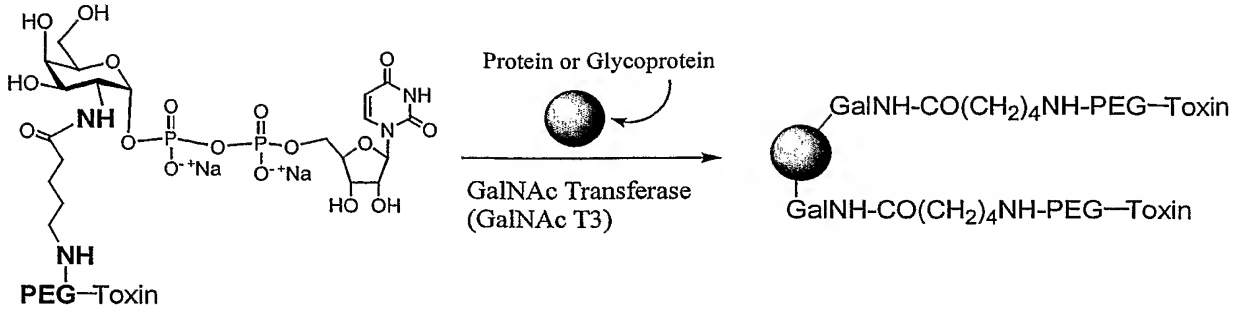
In a further exemplary embodiment, UDP-galactose-PEG is reacted with bovine milk β1,4-galactosyltransferase, thereby transferring the modified galactose to the

appropriate terminal N-acetylglucosamine structure. The terminal GlcNAc residues on the glycopeptide may be produced during expression, as may occur in such expression systems as mammalian, insect, plant or fungus, but also can be produced by treating the glycopeptide with a sialidase and/or glycosidase and/or glycosyltransferase, as required.

In another exemplary embodiment, a GlcNAc transferase, such as GNT1-5, is utilized to transfer PEGylated-GlcN to a terminal mannose residue on a glycopeptide. In a still further exemplary embodiment, an the N- and/or O-linked glycan structures are enzymatically removed from a glycopeptide to expose an amino acid or a terminal glycosyl residue that is subsequently conjugated with the modified sugar. For example, an endoglycanase is used to remove the N-linked structures of a glycopeptide to expose a terminal GlcNAc as a GlcNAc-linked-Asn on the glycopeptide. UDP-Gal-PEG and the appropriate galactosyltransferase is used to introduce the PEG- or PPG-galactose functionality onto the exposed GlcNAc.

In an alternative embodiment, the modified sugar is added directly to the peptide backbone using a glycosyltransferase known to transfer sugar residues to the peptide backbone. This exemplary embodiment is set forth in Scheme 4. Exemplary glycosyltransferases useful in practicing the present invention include, but are not limited to, GalNAc transferases (GalNAc T1-14), GlcNAc transferases, fucosyltransferases, glucosyltransferases, xylosyltransferases, mannosyltransferases and the like. Use of this approach allows the direct addition of modified sugars onto peptides that lack any carbohydrates or, alternatively, onto existing glycopeptides. In both cases, the addition of the modified sugar occurs at specific positions on the peptide backbone as defined by the substrate specificity of the glycosyltransferase and not in a random manner as occurs during modification of a protein's peptide backbone using chemical methods. An array of agents can be introduced into proteins or glycopeptides that lack the glycosyltransferase substrate peptide sequence by engineering the appropriate amino acid sequence into the polypeptide chain.

Scheme 4



In each of the exemplary embodiments set forth above, one or more additional chemical or enzymatic modification steps can be utilized following the conjugation of the modified sugar to the peptide. In an exemplary embodiment, an enzyme (*e.g.*, fucosyltransferase) is used to append a glycosyl unit (*e.g.*, fucose) onto the terminal modified sugar attached to the peptide. In another example, an enzymatic reaction is utilized to "cap" sites to which the modified sugar failed to conjugate. Alternatively, a chemical reaction is utilized to alter the structure of the conjugated modified sugar. For example, the conjugated modified sugar is reacted with agents that stabilize or destabilize its linkage with the peptide component to which the modified sugar is attached. In another example, a component of the modified sugar is deprotected following its conjugation to the peptide. One of skill will appreciate that there is an array of enzymatic and chemical procedures that are useful in the methods of the invention at a stage after the modified sugar is conjugated to the peptide. Further elaboration of the modified sugar-peptide conjugate is within the scope of the invention.

i. Enzymes

1. Glycosyltransferases

Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step-wise fashion, to a protein, glycopeptide, lipid or glycolipid or to the non-reducing end of a growing oligosaccharide. N-linked glycopeptides are synthesized via a transferase and a lipid-linked oligosaccharide donor Dol-PP-$NAG_2Glc_3Man_9$ in an en block transfer followed by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases are known in the art (*see e.g.*, U.S. Patents 5,583,042; 5,879,912; 6,127,153; 6,284,493; 6,331,418; 6,379,933).

The glycosyltransferase to be used in the present invention may be any as long as it can utilize the modified sugar as a sugar donor. Examples of such enzymes include Leloir pathway glycosyltransferase, such as galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, fucosyltransferase, sialyltransferase, mannosyltransferase, xylosyltransferase, glucurononyltransferase and the like.

For enzymatic saccharide syntheses that involve glycosyltransferase reactions, glycosyltransferase can be cloned, or isolated from any source. Many cloned glycosyltransferases are known, as are their polynucleotide sequences. *See, e.g.*, "The WWW Guide To Cloned Glycosyltransferases," (http://www.vei.co.uk/TGN/gt_guide.htm). Glycosyltransferase amino acid sequences and nucleotide sequences encoding glycosyltransferases from which the amino acid sequences can be deduced are also found in various publicly available databases, including GenBank, Swiss-Prot, EMBL, and others.

Glycosyltransferases that can be employed in the methods of the invention include, but are not limited to, galactosyltransferases, fucosyltransferases, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases. Suitable glycosyltransferases include those obtained from eukaryotes, as well as from prokaryotes.

DNA encoding the enzyme glycosyltransferases may be obtained by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures. Screening of mRNA or genomic DNA may be carried out with oligonucleotide probes generated from the glycosyltransferases gene sequence. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with known procedures and used in conventional hybridization assays. In the alternative, glycosyltransferases gene sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the glycosyltransferases gene sequence. *See*, U.S. Pat. No. 4,683,195 to Mullis *et al.* and U.S. Pat. No. 4,683,202 to Mullis.

The glycosyltransferases enzyme may be synthesized in host cells transformed with vectors containing DNA encoding the glycosyltransferases enzyme. A vector is a replicable DNA construct. Vectors are used either to amplify DNA encoding the glycosyltransferases enzyme and/or to express DNA which encodes the glycosyltransferases

enzyme. An expression vector is a replicable DNA construct in which a DNA sequence encoding the glycosyltransferases enzyme is operably linked to suitable control sequences capable of effecting the expression of the glycosyltransferases enzyme in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

a) Fucosyltransferases

In some embodiments, a glycosyltransferase used in the method of the invention is a fucosyltransferase. Fucosyltransferases are known to those of skill in the art. Exemplary fucosyltransferases include enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. Fucosyltransferases that transfer non-nucleotide sugars to an acceptor are also of use in the present invention.

In some embodiments, the acceptor sugar is, for example, the GlcNAc in a Galβ(1→3,4)GlcNAcβ- group in an oligosaccharide glycoside. Suitable fucosyltransferases for this reaction include the Galβ(1→3,4)GlcNAcβ1-α(1→3,4)fucosyltransferase (FTIII E.C. No. 2.4.1.65), which was first characterized from human milk (*see*, Palcic, *et al.*, *Carbohydrate Res.* **190**: 1-11 (1989); Prieels, *et al., J. Biol. Chem.* **256**: 10456-10463 (1981); and Nunez, *et al., Can. J. Chem.* **59**: 2086-2095 (1981)) and the Galβ(1→4)GlcNAcβ-αfucosyltransferases (FTIV, FTV, FTVI) which are found in human serum. FTVII (E.C. No. 2.4.1.65), a sialyl α(2→3)Galβ((1→3)GlcNAcβ fucosyltransferase, has also been characterized. A recombinant form of the Galβ(1→3,4) GlcNAcβ-α(1→3,4)fucosyltransferase has also been characterized (*see*, Dumas, *et al., Bioorg. Med. Letters* **1**: 425-428 (1991) and Kukowska-Latallo, *et al., Genes and Development* **4**: 1288-1303 (1990)). Other exemplary fucosyltransferases include, for example, α1,2 fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, *et al., Eur. J. Biochem.* **191**: 169-176 (1990) or U.S. Patent No. 5,374,655. Cells that are used to produce a fucosyltransferase will also include an enzymatic system for synthesizing GDP-fucose.

b) Galactosyltransferases

In another group of embodiments, the glycosyltransferase is a galactosyltransferase. Exemplary galactosyltransferases include α(1,3) galactosyltransferases (E.C. No. 2.4.1.151, *see, e.g.*, Dabkowski *et al., Transplant Proc.* **25**:2921 (1993) and Yamamoto *et al. Nature* **345**: 229-233 (1990), bovine (GenBank j04989, Joziasse *et al., J. Biol. Chem.* **264**: 14290-14297 (1989)), murine (GenBank m26925; Larsen *et al., Proc. Nat'l. Acad. Sci. USA* **86**: 8227-8231 (1989)), porcine (GenBank L36152; Strahan *et al., Immunogenetics* **41**: 101-105 (1995)). Another suitable α1,3 galactosyltransferase is that which is involved in synthesis of the blood group B antigen (EC 2.4.1.37, Yamamoto *et al., J. Biol. Chem.* **265**: 1146-1151 (1990) (human)).

Also suitable for use in the methods of the invention are β(1,4) galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro *et al., Eur. J. Biochem.* **183**: 211-217 (1989)), human (Masri *et al., Biochem. Biophys. Res. Commun.* **157**: 657-663 (1988)), murine (Nakazawa *et al.,* J. Biochem. **104**: 165-168 (1988)), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl *et al., J. Neurosci. Res.* **38**: 234-242 (1994)). Other suitable galactosyltransferases include, for example, α1,2 galactosyltransferases (from *e.g.*, *Schizosaccharomyces pombe*, Chapell *et al., Mol. Biol. Cell* **5**: 519-528 (1994)).

The production of proteins such as the enzyme GalNAc T_{I-XIV} from cloned genes by genetic engineering is well known. See, eg., U.S. Pat. No. 4,761,371. One method involves collection of sufficient samples, then the amino acid sequence of the enzyme is determined by N-terminal sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by in vitro glycosylation studies of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on acceptor efficiency than other amino acid moieties.

c) Sialyltransferases

Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Cells that produce recombinant sialyltransferases will also produce CMP-sialic acid, which is a sialic acid donor for sialyltransferases. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (*e.g.*, a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et al., Glycobiology* 6: v-xiv (1996)). An exemplary α(2,3)sialyltransferase referred to as α(2,3)sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Galβ1→3Glc disaccharide or glycoside. *See,* Van den Eijnden *et al., J. Biol. Chem.* **256:** 3159 (1981), Weinstein *et al., J. Biol. Chem.* **257:** 13845 (1982) and Wen *et al., J. Biol. Chem.* **267:** 21011 (1992). Another exemplary α2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. *see,* Rearick *et al., J. Biol. Chem.* **254:** 4444 (1979) and Gillespie *et al., J. Biol. Chem.* **267:** 21004 (1992). Further exemplary enzymes include Gal-β-1,4-GlcNAc α-2,6 sialyltransferase (*See*, Kurosawa *et al. Eur. J. Biochem.* **219:** 375-381 (1994)).

Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Galβ1,4GlcNAc-, the most common penultimate sequence underlying the terminal sialic acid on fully sialylated carbohydrate structures (*see,* Table 3).

Table 3: Sialyltransferases which use the Galβ1,4GlcNAc sequence as an acceptor substrate

Sialyltransferase	Source	Sequence(s) formed	Ref.
ST6Gal I	Mammalian	NeuAcI2,6Galβ1,4GlCNAc-	1
ST3Gal III	Mammalian	NeuAcI2,3Galβ1,4GlCNAc- NeuAcI2,3Galβ1,3GlCNAc-	1
ST3Gal IV	Mammalian	NeuAcI2,3Galβ1,4GlCNAc- NeuAcI2,3Galβ1,3GlCNAc-	1
ST6Gal II	Mammalian	NeuAcI2,6Galβ1,4GlCNA	
ST6Gal II	photobacterium	NeuAcI2,6Galβ1,4GlCNAc-	2
ST3Gal V	*N. meningitides* *N. gonorrhoeae*	NeuAcI2,3Galβ1,4GlCNAc-	3

1) Goochee *et al., Bio/Technology* **9**: 1347-1355 (1991)
2) Yamamoto *et al., J. Biochem.* **120**: 104-110 (1996)
3) Gilbert *et al., J. Biol. Chem.* **271**: 28271-28276 (1996)

An example of a sialyltransferase that is useful in the claimed methods is ST3Gal III, which is also referred to as α(2,3)sialyltransferase (EC 2.4.99.6). This enzyme catalyzes the transfer of sialic acid to the Gal of a Galβ1,3GlcNAc or Galβ1,4GlcNAc glycoside (*see, e.g.*, Wen *et al., J. Biol. Chem.* **267**: 21011 (1992); Van den Eijnden *et al., J. Biol. Chem.* **256:** 3159 (1991)) and is responsible for sialylation of asparagine-linked oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an □-linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver (Weinstein *et al., J. Biol. Chem.* **257**: 13845 (1982)); the human cDNA (Sasaki *et al.* (1993) *J. Biol. Chem.* **268**: 22782-22787; Kitagawa & Paulson (1994) *J. Biol. Chem.* **269**: 1394-1401) and genomic (Kitagawa *et al.* (1996) *J. Biol. Chem.* **271**: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression. In a preferred embodiment, the claimed sialylation methods use a rat ST3Gal III.

Other exemplary sialyltransferases of use in the present invention include those isolated from Campylobacter jejuni, including the α(2,3). *See*, e.g, WO99/49051.

Other sialyltransferases, including those listed in Table 4, are also useful in an economic and efficient large-scale process for sialylation of commercially important glycopeptides. As a simple test to find out the utility of these other enzymes, various

amounts of each enzyme (1-100 mU/mg protein) are reacted with asialo-α_1 AGP (at 1-10 mg/ml) to compare the ability of the sialyltransferase of interest to sialylate glycopeptides relative to either bovine ST6Gal I, ST3Gal III or both sialyltransferases. Alternatively, other glycopeptides or glycopeptides, or N-linked oligosaccharides enzymatically released from the peptide backbone can be used in place of asialo-α_1 AGP for this evaluation. Sialyltransferases with the ability to sialylate N-linked oligosaccharides of glycopeptides more efficiently than ST6Gal I are useful in a practical large-scale process for peptide sialylation (as illustrated for ST3Gal III in this disclosure).

d) Other glycosyltransferases

One of skill in the art will understand that other glycosyltransferases can be substituted into similar transferase cycles as have been described in detail for the sialyltransferase. In particular, the glycosyltransferase can also be, for instance, glucosyltransferases, *e.g.*, Alg8 (Stagljov *et al., Proc. Natl. Acad. Sci. USA* **91**: 5977 (1994)) or Alg5 (Heesen *et al., Eur. J. Biochem.* **224**: 71 (1994)).

N-acetylgalactosaminyltransferases are also of use in practicing the present invention. Suitable N-acetylgalactosaminyltransferases include, but are not limited to, α(1,3) N-acetylgalactosaminyltransferase, β(1,4) N-acetylgalactosaminyltransferases (Nagata *et al., J. Biol. Chem.* **267**: 12082-12089 (1992) and Smith *et al., J. Biol Chem.* **269**: 15162 (1994)) and polypeptide N-acetylgalactosaminyltransferase (Homa *et al., J. Biol. Chem.* **268**: 12609 (1993)). Suitable N-acetylglucosaminyltransferases include GnTI (2.4.1.101, Hull *et al., BBRC* **176**: 608 (1991)), GnTII, GnTIII (Ihara *et al., J. Biochem.* **113**: 692 (1993)), GnTIV, and GnTV (Shoreiban *et al., J. Biol. Chem.* **268**: 15381 (1993)), O-linked N-acetylglucosaminyltransferase (Bierhuizen *et al., Proc. Natl. Acad. Sci. USA* **89**: 9326 (1992)), N-acetylglucosamine-1-phosphate transferase (Rajput *et al., Biochem J.* **285**: 985 (1992), and hyaluronan synthase.

Mannosyltransferases are of use to transfer modified mannose moieties. Suitable mannosyltransferases include α(1,2) mannosyltransferase, α(1,3) mannosyltransferase, α(1,6) mannosyltransferase, β(1,4) mannosyltransferase, Dol-P-Man synthase, OCh1, and Pmt1 (*see*, Kornfeld *et al., Annu. Rev. Biochem.* **54**: 631-664 (1985)).

Xylosyltransferases are also useful in the present invention. *See*, for example, Rodgers, *et al.*, Biochem. J., **288**:817-822 (1992); and Elbain, *et al.*, U.S. Patent No., 6,168,937.

Other suitable glycosyltransferase cycles are described in Ichikawa *et al.*, *JACS* **114**: 9283 (1992), Wong *et al., J. Org. Chem.* **57**: 4343 (1992), and Ichikawa *et al.* in CARBOHYDRATES AND CARBOHYDRATE POLYMERS. Yaltami, ed. (ATL Press, 1993).

Prokaryotic glycosyltransferases are also useful in practicing the invention. Such glycosyltransferases include enzymes involved in synthesis of lipooligosaccharides (LOS), which are produced by many gram negative bacteria. The LOS typically have terminal glycan sequences that mimic glycoconjugates found on the surface of human epithelial cells or in host secretions (Preston *et al., Critical Reviews in Microbiology* **23(3)**: 139-180 (1996)). Such enzymes include, but are not limited to, the proteins of the *rfa* operons of species such as *E. coli* and *Salmonella typhimurium*, which include a β1,6 galactosyltransferase and a β1,3 galactosyltransferase (*see, e.g.*, EMBL Accession Nos. M80599 and M86935 (*E. coli*); EMBL Accession No. S56361 (*S. typhimurium*)), a glucosyltransferase (Swiss-Prot Accession No. P25740 (*E. coli*), an β1,2-glucosyltransferase (*rfa*J)(Swiss-Prot Accession No. P27129 (*E. coli*) and Swiss-Prot Accession No. P19817 (*S. typhimurium*)), and an β1,2-N-acetylglucosaminyltransferase (*rfa*K)(EMBL Accession No. U00039 (*E. coli*). Other glycosyltransferases for which amino acid sequences are known include those that are encoded by operons such as *rfa*B, which have been characterized in organisms such as *Klebsiella pneumoniae*, *E. coli*, *Salmonella typhimurium*, *Salmonella enterica*, *Yersinia enterocolitica*, *Mycobacterium leprosum*, and the *rhl* operon of *Pseudomonas aeruginosa*.

Also suitable for use in the present invention are glycosyltransferases that are involved in producing structures containing lacto-N-neotetraose, D-galactosyl-β-1,4-N-acetyl-D-glucosaminyl-β-1,3-D-galactosyl-β-1,4-D-glucose, and the P^k blood group trisaccharide sequence, D-galactosyl-α-1,4-D-galactosyl-β-1,4-D-glucose, which have been identified in the LOS of the mucosal pathogens *Neisseria gonnorhoeae* and *N. meningitidis* (Scholten *et al., J. Med. Microbiol.* **41**: 236-243 (1994)). The genes from *N. meningitidis* and *N. gonorrhoeae* that encode the glycosyltransferases involved in the biosynthesis of these structures have been identified from *N. meningitidis* immunotypes L3 and L1 (Jennings *et al., Mol. Microbiol.* **18**: 729-740 (1995)) and the *N. gonorrhoeae* mutant F62 (Gotshlich, *J. Exp. Med.* **180**: 2181-2190 (1994)). In *N. meningitidis*, a locus consisting of three genes, *lgtA*, *lgtB* and *lg E*, encodes the glycosyltransferase enzymes required for addition of the last three of the sugars in the lacto-*N*-neotetraose chain (Wakarchuk *et al., J. Biol. Chem.* **271**: 19166-73 (1996)). Recently the enzymatic activity of the *lgtB* and *lgtA* gene product was

demonstrated, providing the first direct evidence for their proposed glycosyltransferase function (Wakarchuk *et al., J. Biol. Chem.* **271(45)**: 28271-276 (1996)). In *N. gonorrhoeae*, there are two additional genes, *lgtD* which adds β-D-GalNAc to the 3 position of the terminal galactose of the lacto-*N*-neotetraose structure and *lgtC* which adds a terminal α-D-Gal to the lactose element of a truncated LOS, thus creating the P^k blood group antigen structure (Gotshlich (1994), *supra.*). In *N. meningitidis*, a separate immunotype L1 also expresses the P^k blood group antigen and has been shown to carry an *lgtC* gene (Jennings *et al.,* (1995), *supra.*). *Neisseria* glycosyltransferases and associated genes are also described in USPN 5,545,553 (Gotschlich). Genes for α1,2-fucosyltransferase and α1,3-fucosyltransferase from *Helicobacter pylori* has also been characterized (Martin *et al., J. Biol. Chem.* **272**: 21349-21356 (1997)). Also of use in the present invention are the glycosyltransferases of *Campylobacter jejuni* (*see*, for example, http://afmb.cnrs-mrs.fr/~pedro/CAZY/gtf_42.html).

2. Sulfotransferases

The invention also provides methods for producing peptides that include sulfated molecules, including, for example sulfated polysaccharides such as heparin, heparan sulfate, carragenen, and related compounds. Suitable sulfotransferases include, for example, chondroitin-6-sulphotransferase (chicken cDNA described by Fukuta *et al., J. Biol. Chem.* **270**: 18575-18580 (1995); GenBank Accession No. D49915), glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 1 (Dixon *et al., Genomics* **26**: 239-241 (1995); UL18918), and glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 2 (murine cDNA described in Orellana *et al., J. Biol. Chem.* **269**: 2270-2276 (1994) and Eriksson *et al., J. Biol. Chem.* **269**: 10438-10443 (1994); human cDNA described in GenBank Accession No. U2304).

3. Cell-Bound Glycosyltransferases

In another embodiment, the enzymes utilized in the method of the invention are cell-bound glycosyltransferases. Although many soluble glycosyltransferases are known (see, for example, U.S. Pat. No. 5,032,519), glycosyltransferases are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been recognized that these surface transferases maintain catalytic activity under physiological

conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular recognition (Roth, MOLECULAR APPROACHES to SUPRACELLULAR PHENOMENA, 1990).

Methods have been developed to alter the glycosyltransferases expressed by cells. For example, Larsen *et al.*, *Proc. Natl. Acad. Sci. USA* **86**: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose:.β.-D-galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase was transfected into COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

Francisco *et al., Proc. Natl. Acad. Sci. USA* **89**: 2713-2717 (1992), disclose a method of anchoring β-lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β-lactamase sequence is produced resulting in an active surface bound β-lactamase molecule. However, the Francisco method is limited only to procaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning.

4. Fusion Proteins

In other exemplary embodiments, the methods of the invention utilize fusion proteins that have more than one enzymatic activity that is involved in synthesis of a desired glycopeptide conjugate. The fusion polypeptides can be composed of, for example, a catalytically active domain of a glycosyltransferase that is joined to a catalytically active domain of an accessory enzyme. The accessory enzyme catalytic domain can, for example, catalyze a step in the formation of a nucleotide sugar that is a donor for the glycosyltransferase, or catalyze a reaction involved in a glycosyltransferase cycle. For example, a polynucleotide that encodes a glycosyltransferase can be joined, in-frame, to a polynucleotide that encodes an enzyme involved in nucleotide sugar synthesis. The resulting fusion protein can then catalyze not only the synthesis of the nucleotide sugar, but also the transfer of the sugar moiety to the acceptor molecule. The fusion protein can be two or more cycle enzymes linked into one expressible nucleotide sequence. In other embodiments the fusion protein includes the catalytically active domains of two or more glycosyltransferases. See, for example, 5,641,668. The modified glycopeptides of the present invention can be

readily designed and manufactured utilizing various suitable fusion proteins (*see*, for example, PCT Patent Application PCT/CA98/01180, which was published as WO 99/31224 on June 24, 1999.)

5. Immobilized Enzymes

In addition to cell-bound enzymes, the present invention also provides for the use of enzymes that are immobilized on a solid and/or soluble support. In an exemplary embodiment, there is provided a glycosyltransferase that is conjugated to a PEG via an intact glycosyl linker according to the methods of the invention. The PEG-linker-enzyme conjugate is optionally attached to solid support. The use of solid supported enzymes in the methods of the invention simplifies the work up of the reaction mixture and purification of the reaction product, and also enables the facile recovery of the enzyme. The glycosyltransferase conjugate is utilized in the methods of the invention. Other combinations of enzymes and supports will be apparent to those of skill in the art.

Purification of Peptide Conjugates

The products produced by the above processes can be used without purification. However, it is usually preferred to recover the product. Standard, well-known techniques for recovery of glycosylated saccharides such as thin or thick layer chromatography, column chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a reverse osmotic membrane, or one or more column chromatographic techniques for the recovery as is discussed hereinafter and in the literature cited herein. For instance, membrane filtration wherein the membranes have molecular weight cutoff of about 3000 to about 10,000 can be used to remove proteins such as glycosyl transferases. Nanofiltration or reverse osmosis can then be used to remove salts and/or purify the product saccharides (*see, e.g.,* WO 98/15581). Nanofilter membranes are a class of reverse osmosis membranes that pass monovalent salts but retain polyvalent salts and uncharged solutes larger than about 100 to about 2,000 Daltons, depending upon the membrane used. Thus, in a typical application, saccharides prepared by the methods of the present invention will be retained in the membrane and contaminating salts will pass through.

If the modified glycoprotein is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a

commercially available protein concentration filter, followed by separating the polypeptide variant from other impurities by one or more steps selected from immunoaffinity chromatography, ion-exchange column fractionation (*e.g.*, on diethylaminoethyl (DEAE) or matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (*e.g.*, silica gel with appended aliphatic groups), gel filtration using, *e.g.*, Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the polypeptide, and ethanol or ammonium sulfate precipitation.

Modified glycopeptides produced in culture are usually isolated by initial extraction from cells, enzymes, etc., followed by one or more concentration, salting-out, aqueous ion-exchange, or size-exclusion chromatography steps. Additionally, the modified glycoprotein may be purified by affinity chromatography. Finally, HPLC may be employed for final purification steps.

A protease inhibitor, *e.g.*, methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

Within another embodiment, supernatants from systems which sproduce the modified glycopeptide of the invention are first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For example, a suitable affinity matrix may comprise a ligand for the peptide, a lectin or antibody molecule bound to a suitable support. Alternatively, an anion-exchange resin may be employed, for example, a matrix or substrate having pendant DEAE groups. Suitable matrices include acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Alternatively, a cation-exchange step may be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are particularly preferred.

Finally, one or more RP-HPLC steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, may be employed to further purify a polypeptide variant composition. Some or all of the foregoing purification

steps, in various combinations, can also be employed to provide a homogeneous modified glycoprotein.

The modified glycopeptide of the invention resulting from a large-scale fermentation may be purified by methods analogous to those disclosed by Urdal *et al., J. Chromatog.* **296**: 171 (1984). This reference describes two sequential, RP-HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column. Alternatively, techniques such as affinity chromatography may be utilized to purify the modified glycoprotein.

Pharmaceutical Compositions

In another aspect, the invention provides a pharmaceutical composition. The pharmaceutical composition includes a pharmaceutically acceptable diluent and a covalent conjugate between a non-naturally-occurring, water-soluble polymer, therapeutic moiety or biomolecule and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group interposed between and covalently linked to both the peptide and the polymer, therapeutic moiety or biomolecule.

Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, *see,* Langer, *Science* **249**:1527-1533 (1990).

The pharmaceutical compositions may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.,* polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

Commonly, the pharmaceutical compositions are administered parenterally, *e.g.*, intravenously. Thus, the invention provides compositions for parenteral administration

which comprise the compound dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, *e.g.*, water, buffered water, saline, PBS and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like.

These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9 and most preferably from 7 and 8.

In some embodiments the glycopeptides of the invention can be incorporated into liposomes formed from standard vesicle-forming lipids. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* **9**: 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028. The targeting of liposomes using a variety of targeting agents (*e.g.*, the sialyl galactosides of the invention) is well known in the art (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044).

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid-derivatized glycopeptides of the invention.

Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The carbohydrates of the invention may be attached to a lipid molecule before the liposome is formed using methods known to those of skill in the art (*e.g.*, alkylation or acylation of a hydroxyl group present on the carbohydrate with a long chain alkyl halide or with a fatty acid, respectively). Alternatively, the liposome may be fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion, which is firmly embedded and anchored in the membrane. It must also have a reactive portion, which is chemically available on the aqueous surface of the liposome. The reactive portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent or carbohydrate, which is added later. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking

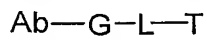
the connector molecule which is in the membrane with the target agent or carbohydrate which is extended, three dimensionally, off of the vesicle surface.

The compounds prepared by the methods of the invention may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient suspected of having an inflammation. For this use, the compounds can be labeled with ^{125}I, ^{14}C, or tritium.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. A compound having the formula:



wherein

Ab is an antibody;

G is an intact glycosyl linking group covalently joining Ab to L;

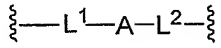
L is a bond or a spacer moiety covalently joining G to T; and

T is a toxin.

2. The compound according to claim 1, wherein said linker moiety is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl moieties.

3. The compound according to claim 2, wherein said linker moiety comprises a poly(ethylene glycol) moiety.

4. The compound according to claim 1, wherein L has the formula:



wherein

L^1 is a bond or a linker moiety covalently joining S to A;

A is an amplifier moiety; and

L^2 is a bond or a spacer moiety covalently adjoining A to T.

5. The compound according to claim 4, wherein said amplifier moiety is a polyamine moiety.

6. The compound according to claim 5, wherein said polyamine moiety is a dendrimer.

7. The compound according to claim 4, having the formula:

wherein

PEG is a straight- or branched-chain poly(ethylene glycol);

m is an integer from 1 to 6; and

n is an integer from 1 to 1,000.

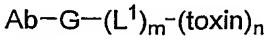
8. The compound according to claim 4, having the formula:

wherein

m is an integer from 1 to 6; and

n is an integer from 1 to 1,000.

9. The compound according to claim 4, having the formula:

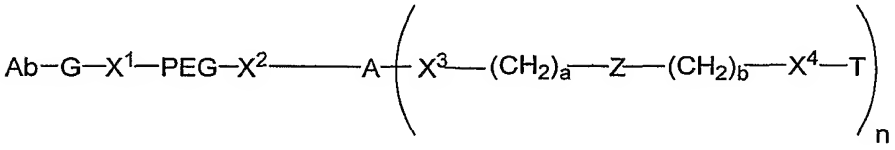


wherein

m is an integer from 1 to 6; and

n is an integer from 1 to 1,000.

10. The compound according to claim 1, having the formula:



wherein

X^1, X^2 and X^4 are linking groups and are members selected from the group consisting of O, S, NH, $(CH_2)_q$-NH, NH-$(CH_2)_q$, NH-C(O)-O, O-C(O)-NH, $(CH_2)_q$-NH-C(O)-O, O-C(O)-NH-$(CH_2)_q$, C(O)-O, O-C(O), $(CH_2)_q$-NH-C(O), C(O)-NH-$(CH_2)_q$, NH-C(S), and C(S)-NH

and wherein

A is an amplifier moiety;

Z is a bond cleaved by a metabolic/physiological process;

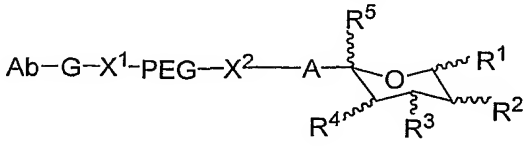
n is an integer from 1 to 1,000;

a is an integer from 1 to 10;

b is an integer from 1 to 10; and

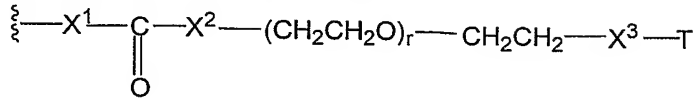
q is and integer from 0 to 20.

11. The compound according to claim **1**, having the formula:



wherein

at least one of R^1, R^2, R^3, R^4, R^5, is :



wherein

r is an integer from 1 to 2,500;

Z^1 is selected from the group consisting of O, S, and NH;

Z^2 is selected from the group consisting of NH, and NH-$(CH_2)_q$;

and

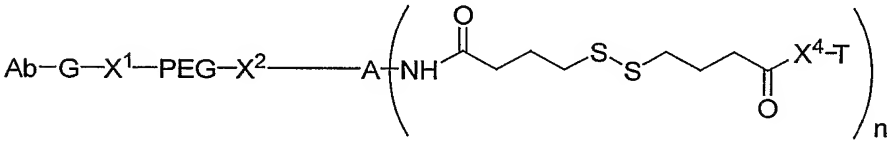
X^1, X^2 and X^3 are linking groups and are members selected from the group consisting of O, S, NH, $(CH_2)_q$-NH, NH-$(CH_2)_q$, NH-C(O)-O, O-C(O)-NH, $(CH_2)_q$-NH-C(O)-O, O-C(O)-NH-$(CH_2)_q$, C(O)-O, O-C(O), $(CH_2)_q$-NH-C(O), C(O)-NH-$(CH_2)_q$, NH-C(S), and C(S)-NH

wherein

n is an integer from 1 to 1,000; and

q is an integer from 0 to 20.

12. The compound according to claim **1**, having the formula:



wherein

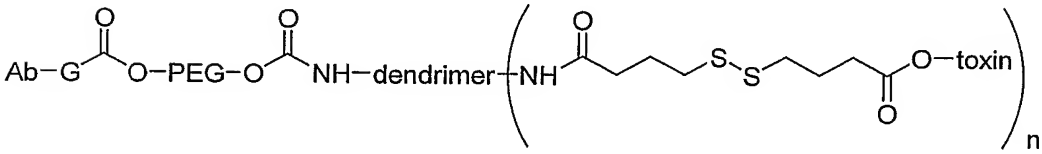
X^1, X^2 and X^4 are linking groups and are members selected from the group consisting of O, S, NH, $(CH_2)_q$-NH, NH-$(CH_2)_q$, NH-C(O)-O, O-C(O)-NH, $(CH_2)_q$-NH-C(O)-O, O-C(O)-NH-$(CH_2)_q$, C(O)-O, O-C(O), $(CH_2)_q$-NH-C(O), C(O)-NH-$(CH_2)_q$, NH-C(S), and C(S)-NH

wherein

n is an integer from 1 to 1,000; and

q is an integer from 0 to 20.

13. The compound according to claim 12, having the formula:



14. A compound having the formula:

$$S—L—T$$

wherein

S is a nucleotide sugar

L is a bond or a spacer moiety covalently joining S to T; and

T is a toxin moiety.

15. The compound according to claim 14, wherein said spacer moiety is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl moieties.

16. The compound according to claim 15, wherein said spacer moiety comprises a poly(ethylene glycol) moiety.

17. The compound according to claim 14, wherein L has the formula:

$$\{\!\!\{—L^1—A—L^2—\}\!\!\}$$

wherein

L^1 is a bond or a spacer moiety covalently joining S to A;

A is an amplifier moiety; and

L^2 is a bond or a spacer moiety covalently joining A to T.

18. The compound according to claim 17, wherein said amplifier moiety is a polyamine moiety.

19. The compound according to claim 18, wherein said polyamine moiety is a dendrimer.

20. The compound according to claim 17, having the formula:

$$S—(PEG)_m-(toxin)_n$$

wherein

PEG is a straight- or branched-chain poly(ethylene glycol);

m is an integer from 1 to 6; and

n is an integer from 1 to 1,000.

21. The compound according to claim 17, having the formula:

$$S—L^1-(dendrimer)_m-(L^2-toxin)_n$$

wherein

m is an integer from 1 to 6; and

n is an integer from 1 to 1,000.

22. The compound according to claim 17, having the formula:

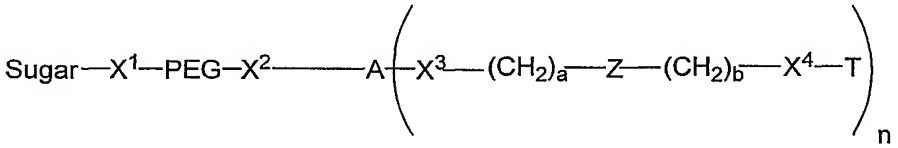
$$S-(L^1)_m-(toxin)_n$$

wherein

m is an integer from 1 to 6; and

n is an integer from 1 to 1,000.

23. The compound according to claim 22, having the formula:



wherein

X^1, X^2 and X^3 are linking groups and are members selected from the group consisting of O, S, $NH(CH_2)_q$-NH, NH-$(CH_2)_q$, NH-C(O)-O, O-C(O)-NH, $(CH_2)_q$-NH-C(O)-O, O-C(O)-NH-$(CH_2)_q$, C(O)-O, O-C(O), $(CH_2)_q$-NH-C(O), C(O)-NH-$(CH_2)_q$, NH-C(S), and C(S)-NH

and wherein

A is an amplifier moiety;

Z is a bond cleaved by a metabolic/physiological process;

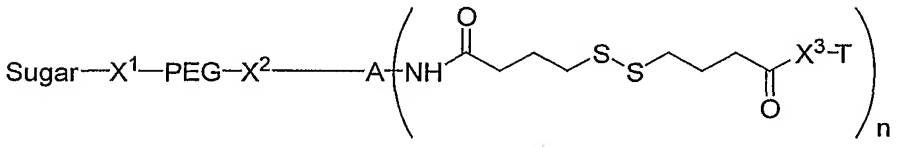
n is an integer from 1 to 1,000;

a is an integer from 1 to 10;

b is an integer from 1 to 10; and

q is and integer from 0 to 20.

24. The compound according to claim 14, having the formula:



wherein

X^1, X^2 and X^3 are linking groups and are members selected from the group consisting of O, S, $NH(CH_2)_q$-NH, NH-$(CH_2)_q$, NH-C(O)-O,

O-C(O)-NH, $(CH_2)_q$-NH-C(O)-O, O-C(O)-NH-$(CH_2)_q$, C(O)-O, O-C(O), $(CH_2)_q$-NH-C(O), C(O)-NH-$(CH_2)_q$, NH-C(S), and C(S)-NH

wherein

q is an integer from 0 to 20.

25. The compound according to claim 24, having the formula:

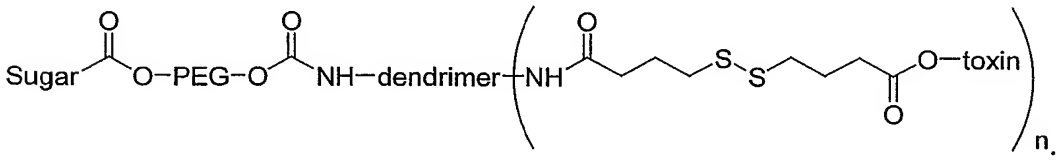


Figure 1. N-linked Glycoprotein Structures.

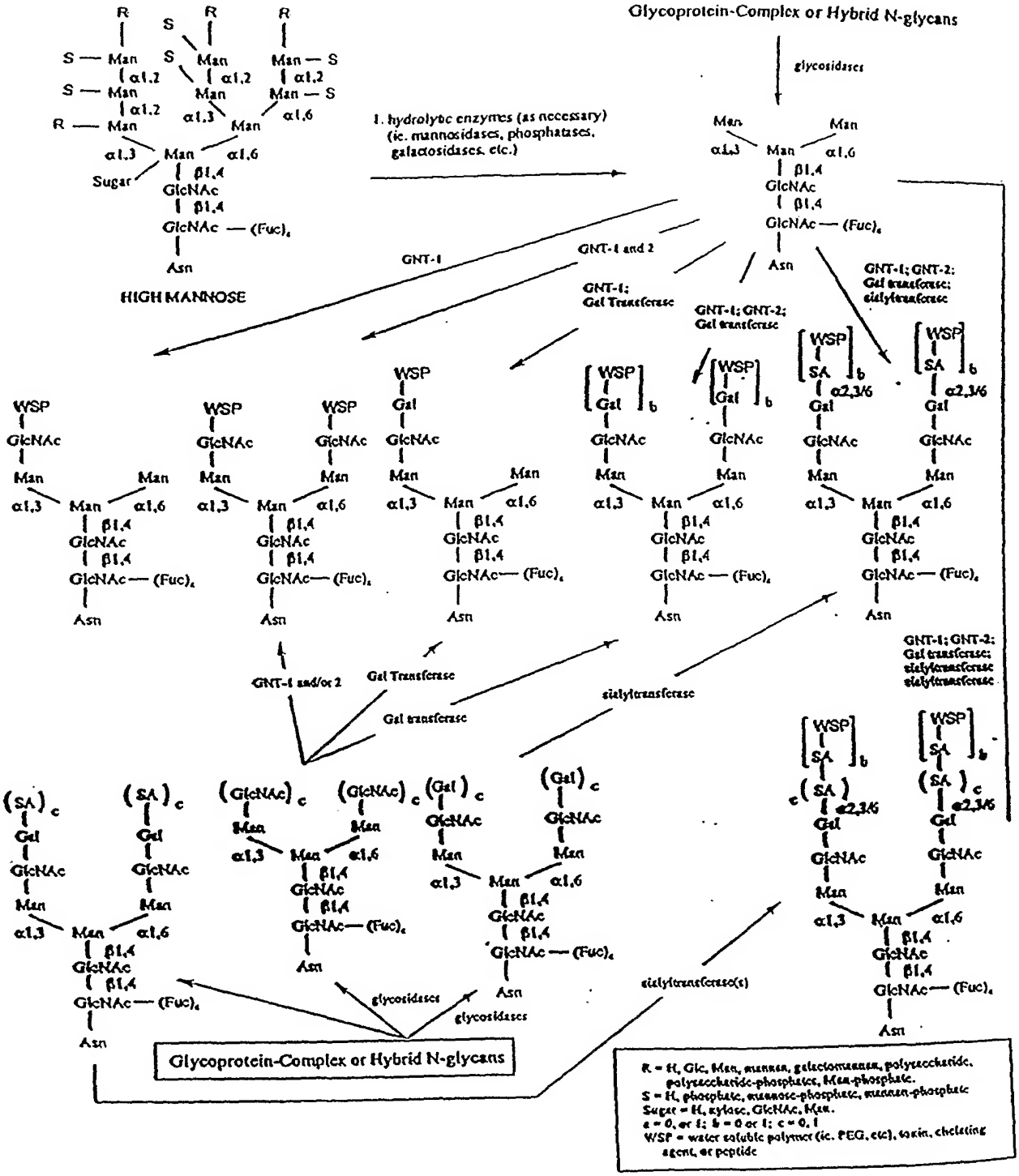


FIG. 1

Scheme 2.

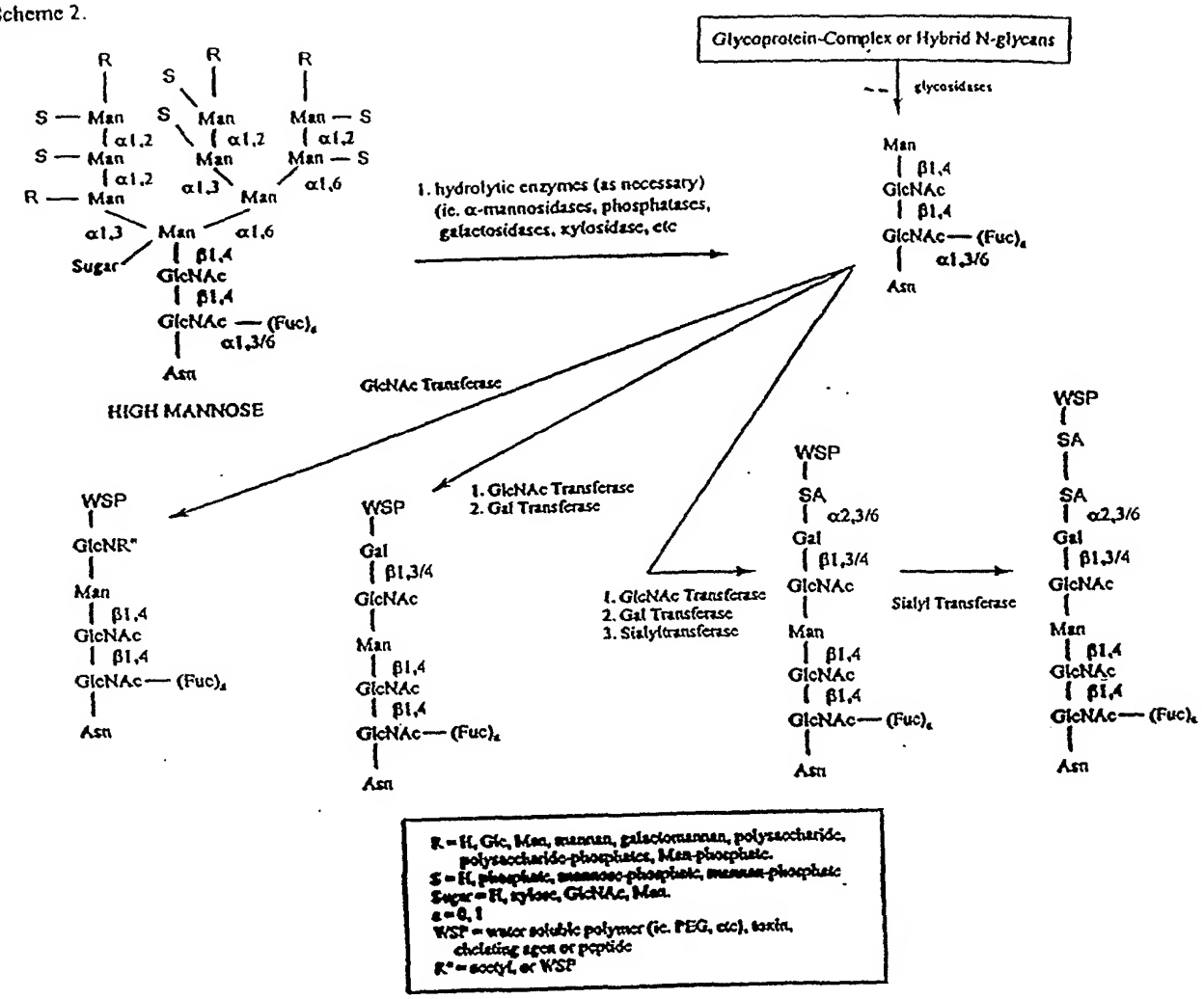


FIG. 2

Scheme 3.

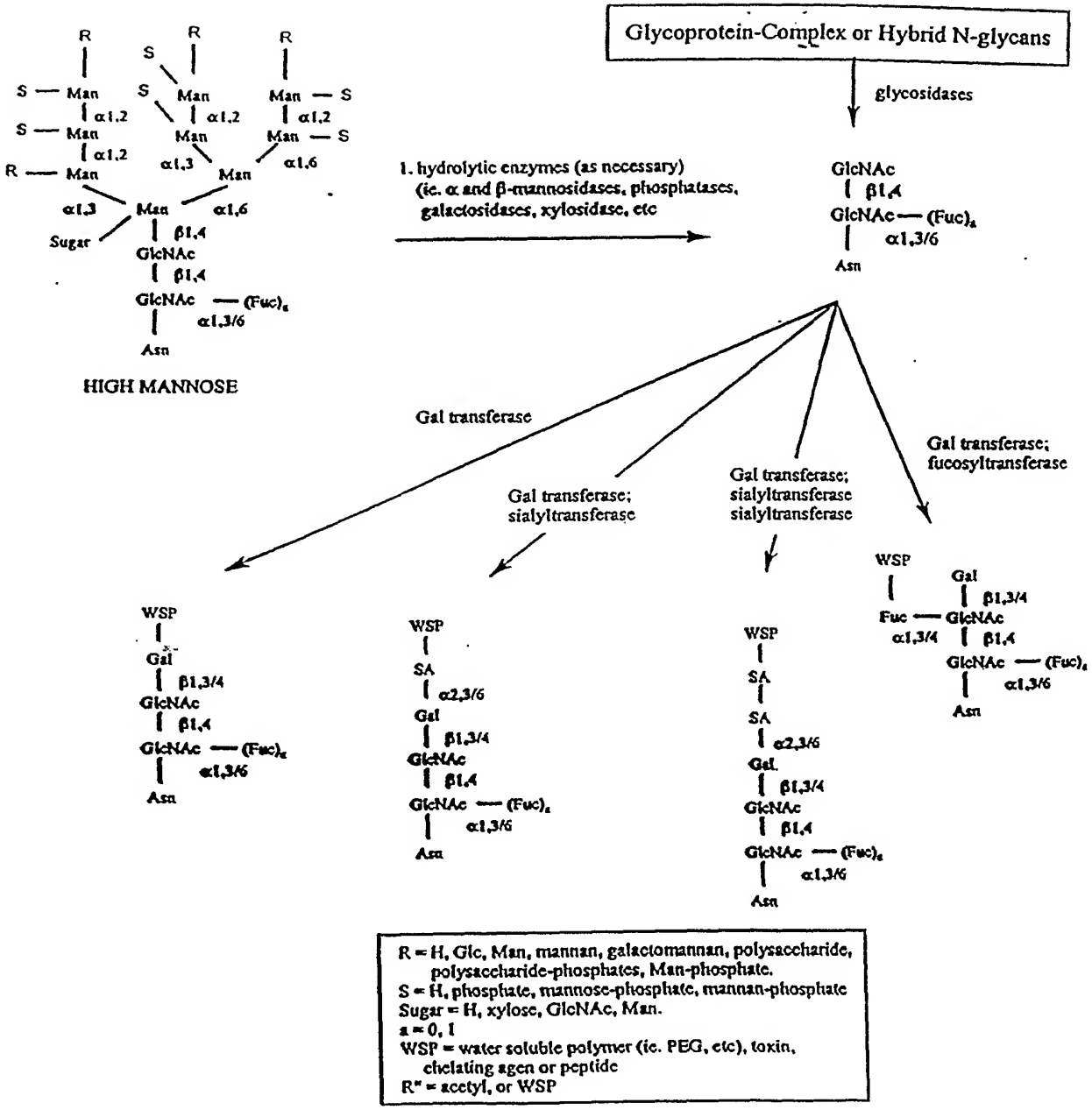


FIG. 3

Scheme 4.

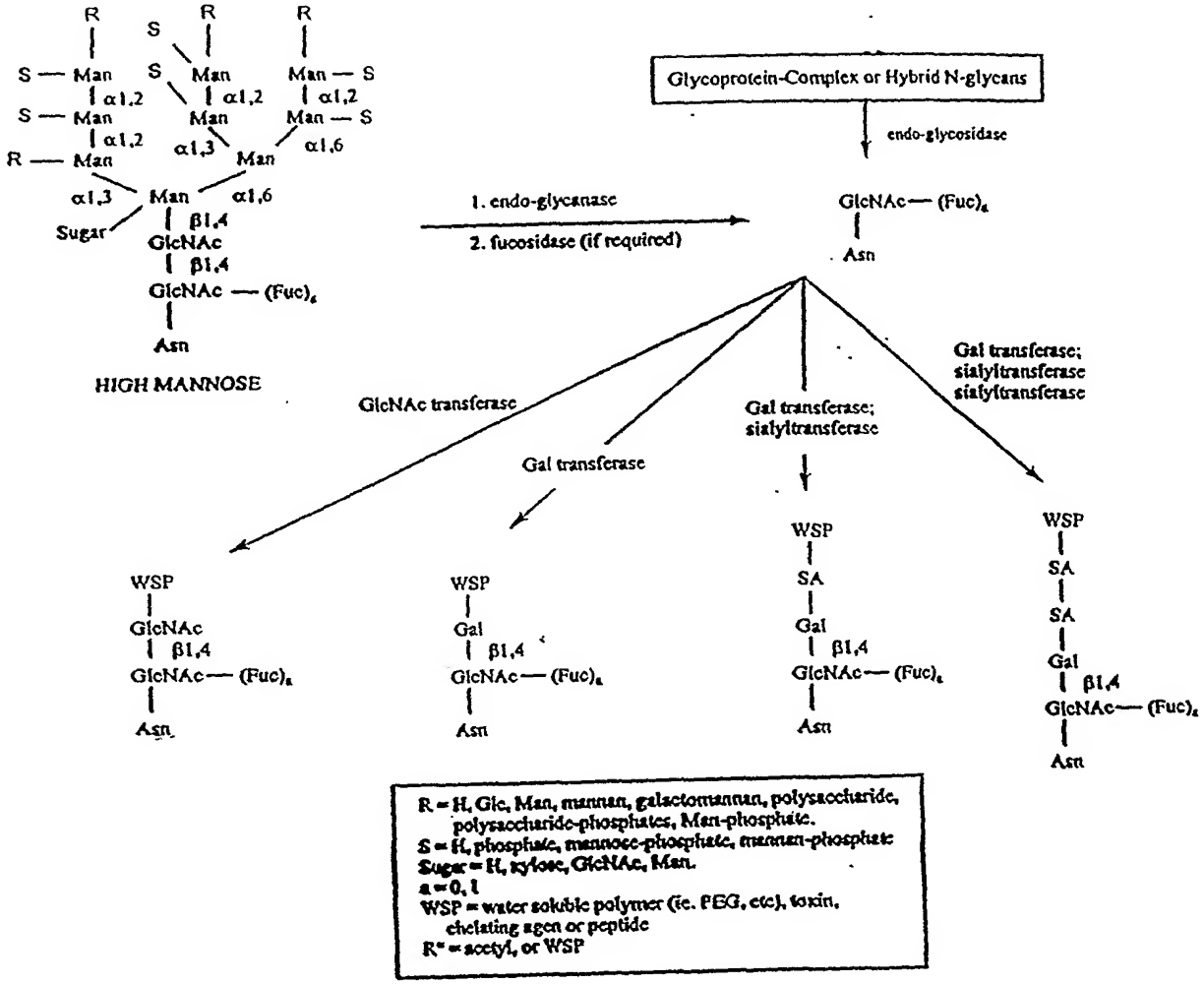


FIG. 4

Figure 5. N-linked Glycoprotein Structures.

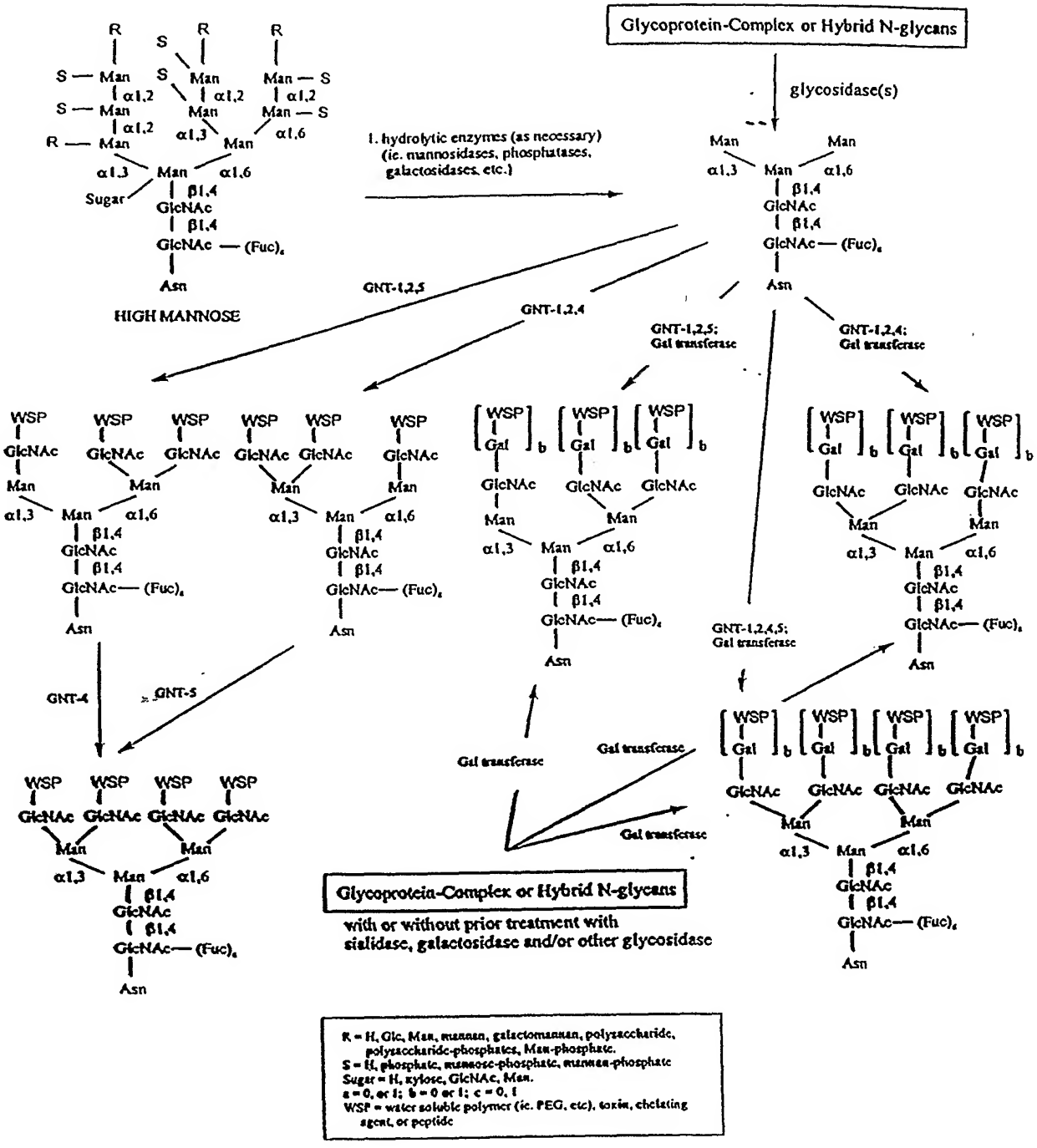


FIG. 5

Figure 6. N-linked Glycoprotein Structures.

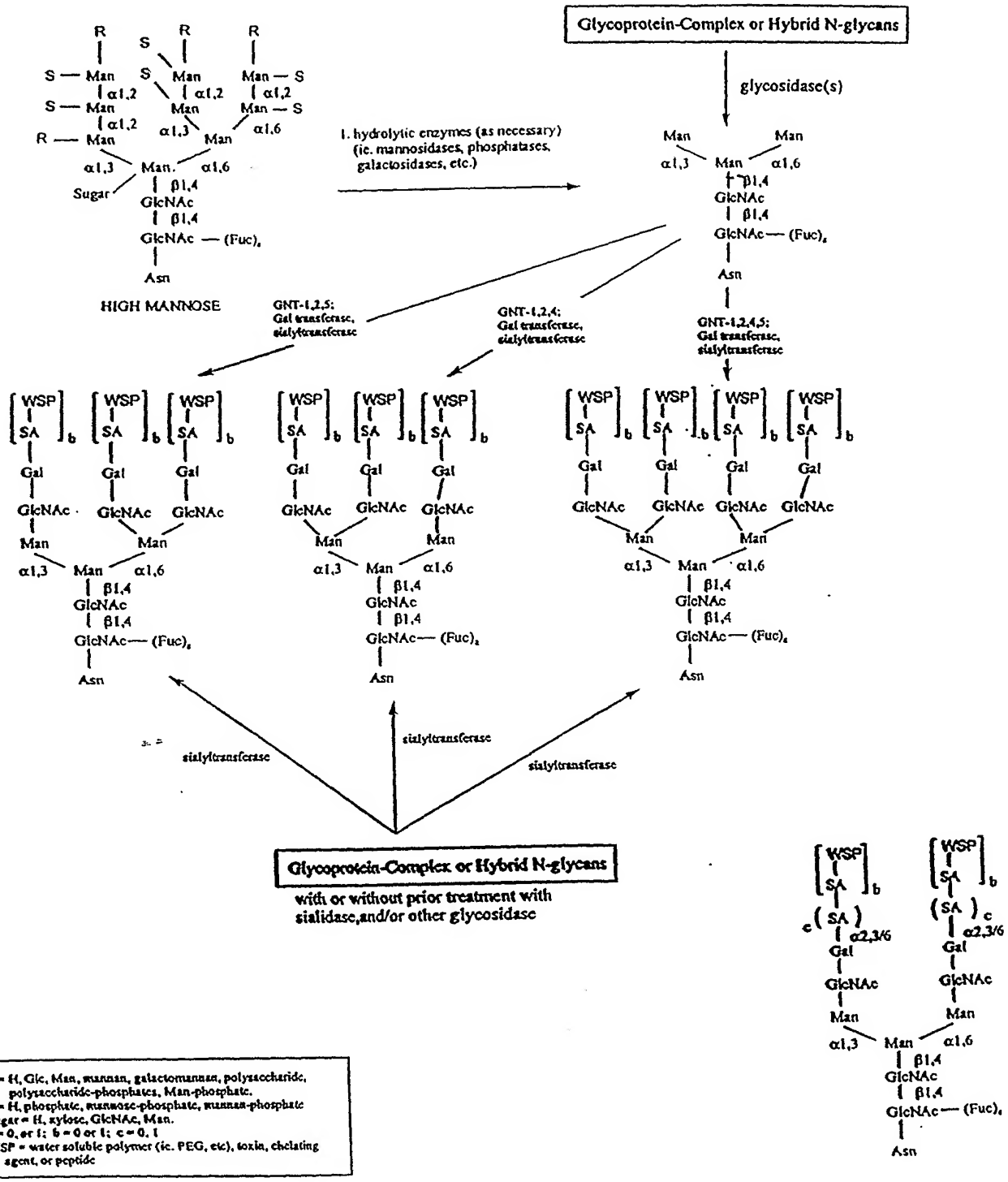


FIG. 6

Figure 7. N-linked Glycoprotein Structures.

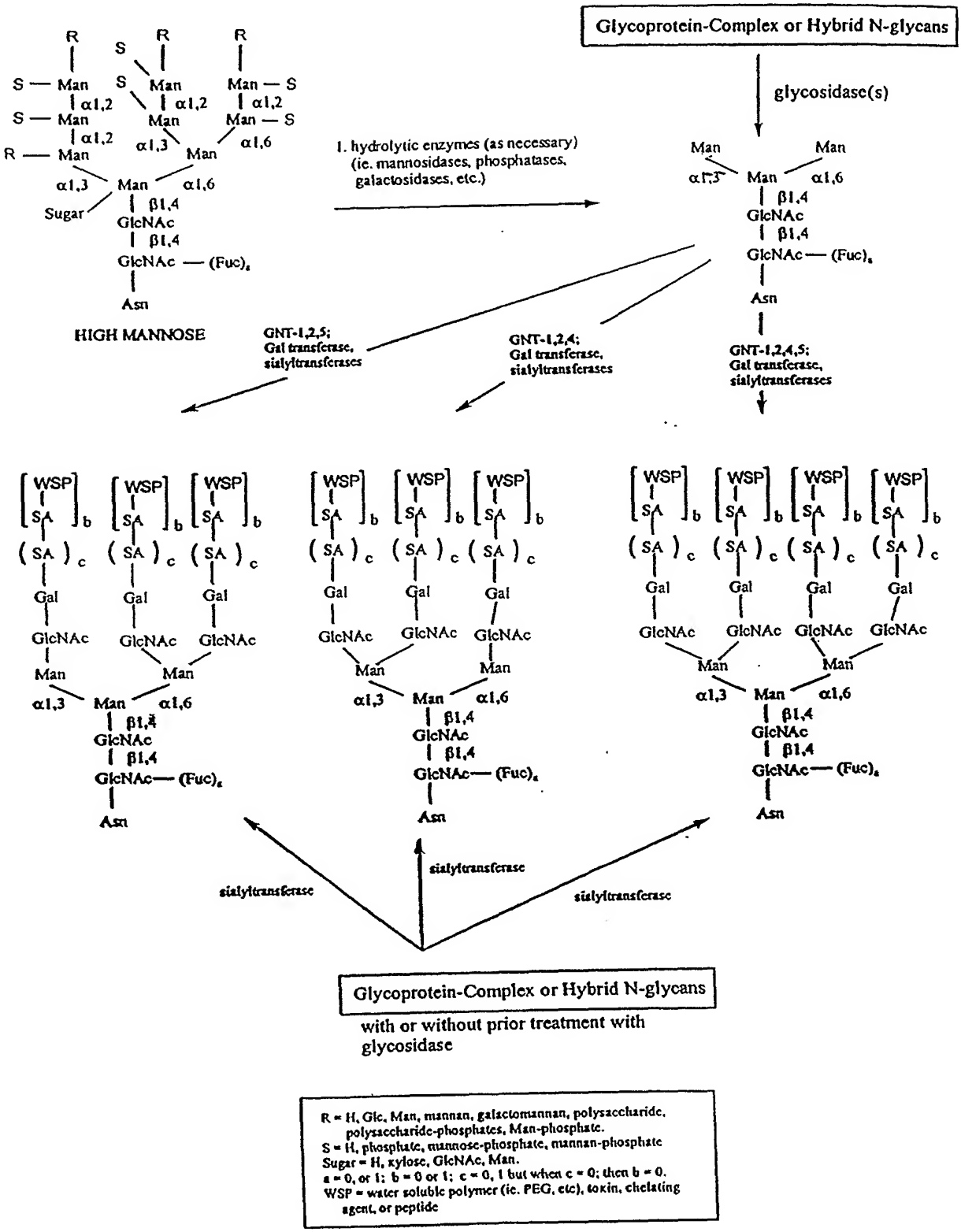


FIG. 7

Scheme 8.

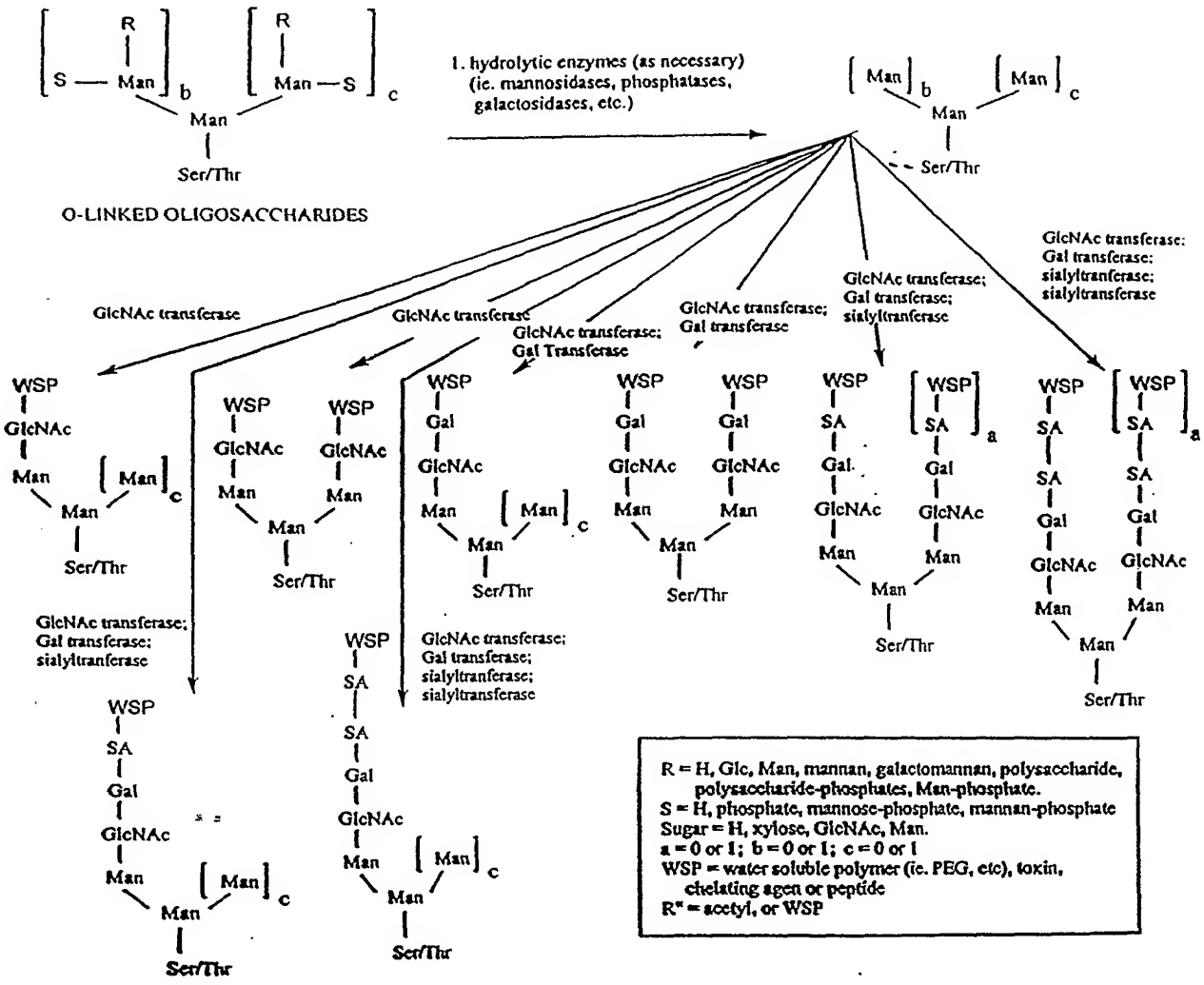


FIG. 8

Scheme 9.

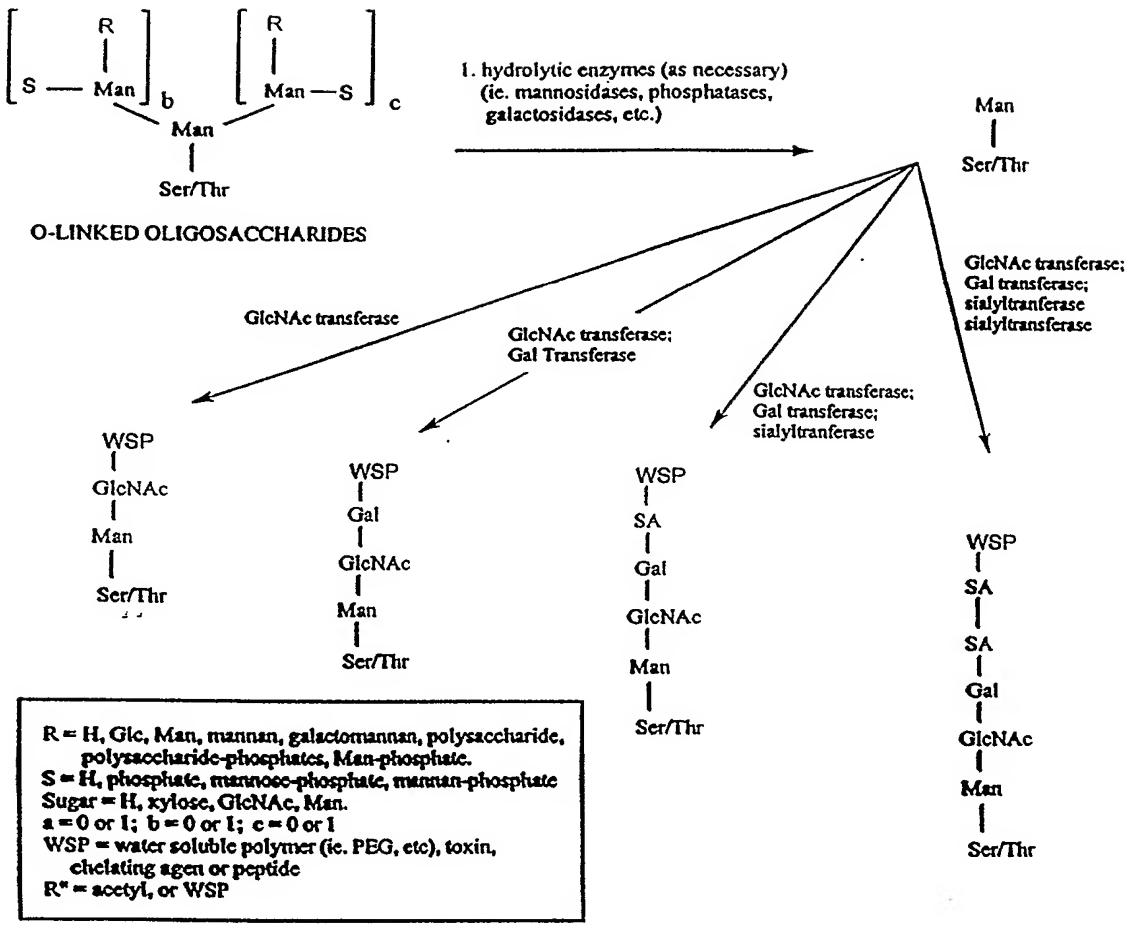


FIG. 9

Scheme 10.

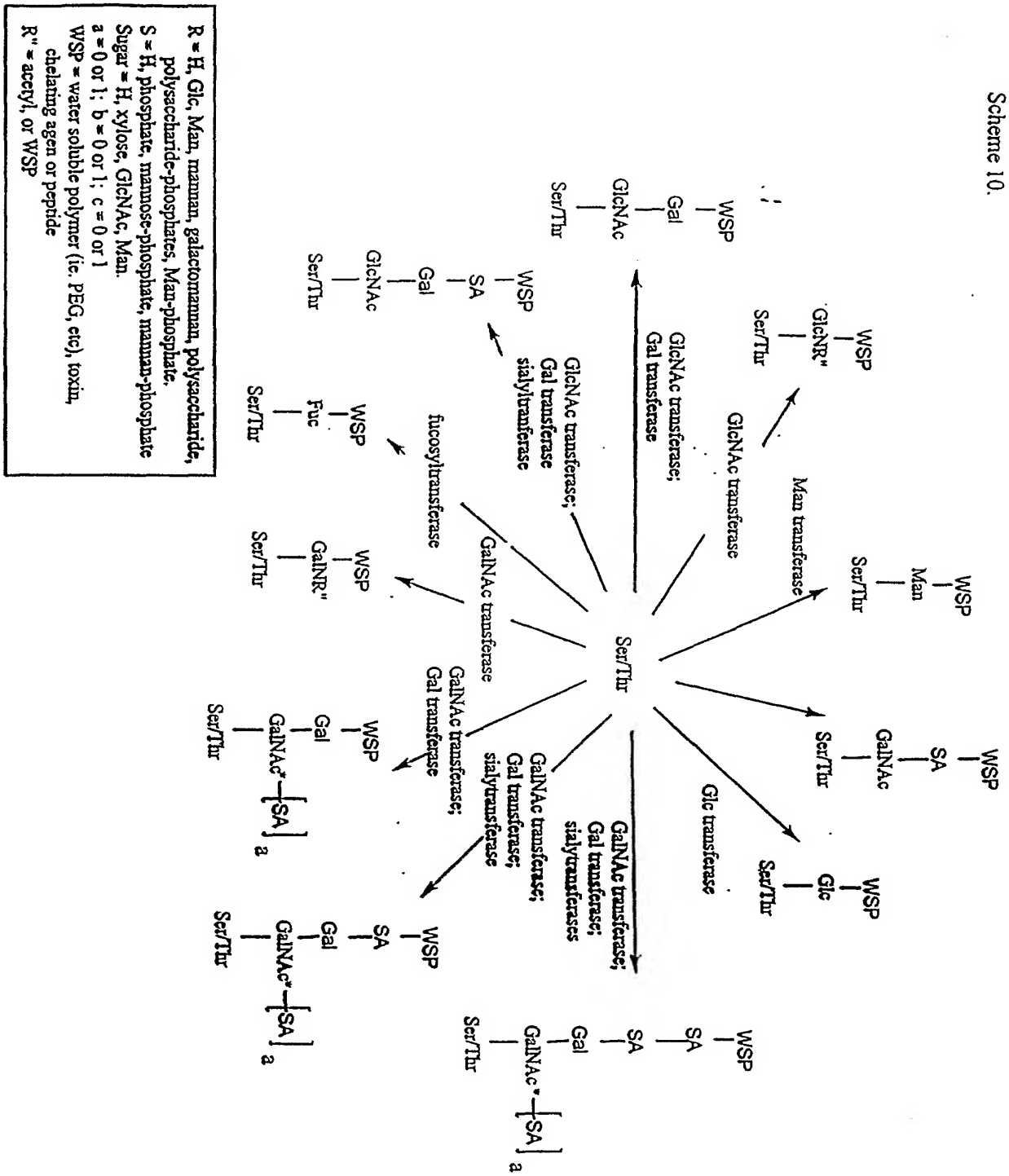


FIG. 10

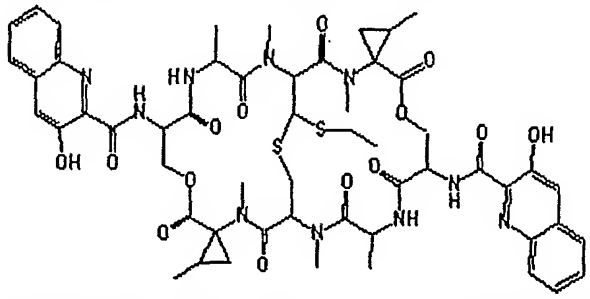
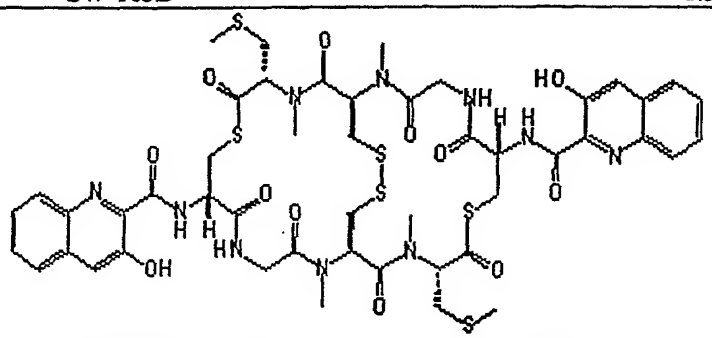
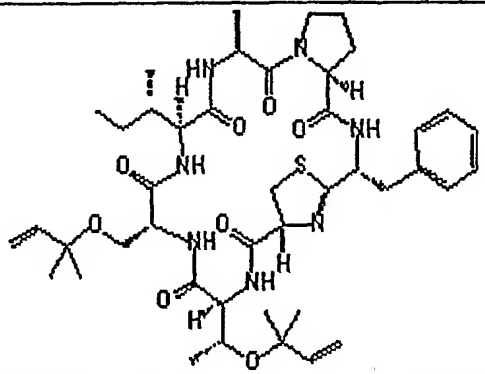
Chemical Structure				
Toxin Name/ Source/ Alternate ID	**CAS RN / Analogs**	**Indication/ Toxicity**	**Mechanism**	**Activity (IC50 nM); Tumor Type**
				
SW-163E/ *Streptomyces* sp SNA 15896/ SW-163E	260794-24-9; 260794-25-0/ SW-163C; SW-163A; SW-163B	Cancer and Antibacterial/ low toxicity (mice ip)	not reported	0.3 P388 0.2 A2780 0.4 KB 1.6 colon 1.3 HL-60
				
Thiocoraline/ [2-4] *Micromonospora marina* (actinomycete)	173046-02-1	Breast Cancer; Melanoma; Non-small lung cancer / not reported	DNA Polymerase alpha inhibitor (blocks cell progression from G1 to S)	lung, colon, CNS melanoma
				
Trunkamide A[1]**/** *Lissoclinum* sp (aascidian)	181758-83-8	Cancer/ not reported	not reported	cell culture (IC50 in micrograms/mL); 0.5 P388; 0.5 A549;

FIG. 11A

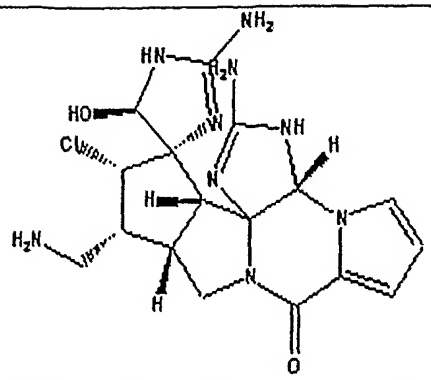
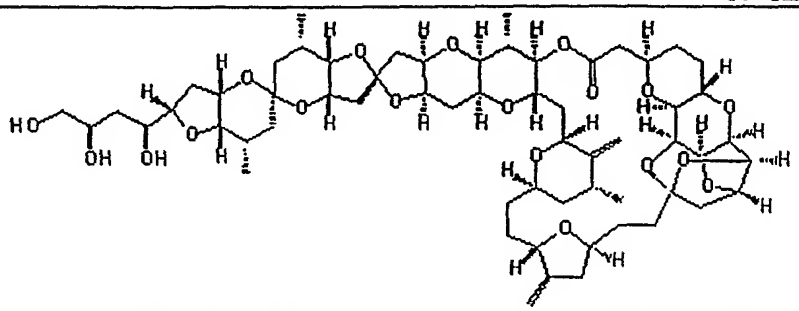
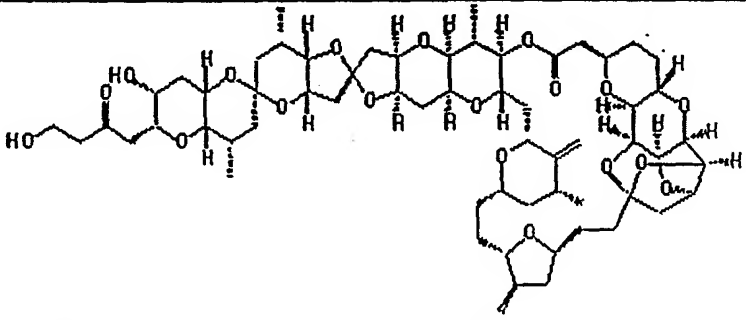
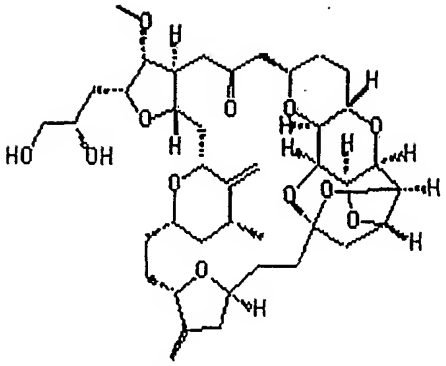
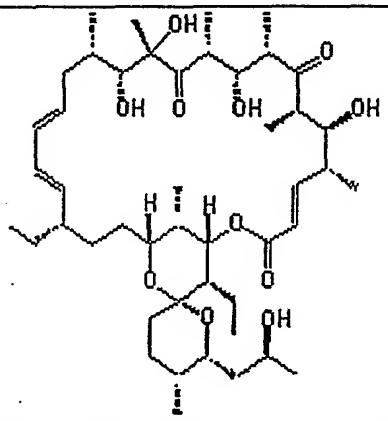
				0.5 HT-29; 1.0 MEL-28
				
Palauamine[2]/ *Stylotella agminata* (sponge)	148717-58-2	Lung cancer/ LD50 (i.p. in mice) is 13 mg/Kg	not reported	cell culture (IC50 in micrograms/mL); 0.1 P388 0.2 A549 (lung) 2 HT-29 (colon) 10 KB
				
Halichondrin B/ *Halichondria Okadai, Axinell Carteri and Phankell carteri* (sponges)/ NSC-609385	103614-76-2/ isohomohalic hondrin B	cancer/ myelotoxicity dose limiting (dogs, rats)	antitubulin; cell cycle inhibitor (inhibits GTP binding to tubulin)	NCI tumor panel; GI(50) from 50 nM to 0.1 nM; LC50's from 40 μM to 0.1 nM (many 0.1 to 25 nM)
				
Isohomo-halichondrin B/ *Halichondria Okadai, Axinell Carteri and Phankell carteri* (sponges)/ NSC-650467	157078-48-3/ halichondrin B	melanoma, lung, CNS, colon, ovary/ not reported	antitubulin; cell cycle inhibitor (inhibits GTP binding to tubulin)	IC50's in 0.1 nM range (NCI tumor panel)

FIG. 11B



Halichondrin B analogs/ semi-synthetic starting from *Halichondria Okadai, Axinell Carteri and Phankell carteri* (sponges)/ ER-076349; ER-086526; B-1793; E-7389	253128-15-3/ ER-076349; ER-086526; B-1793; E-7389	solid tumors/ not reported	tubulin binding agent; disruption of mitotic spindles	cell culture (not reported); animal models active (tumor regression observed) in lymphoma, colon (multi-drug resistant).



NK-130119/ *Streptomyces bottropensis*/ NK-130119	132707-68-7	antifungal and anticancer/ not reported	not reported	25 ng/mL colon 8.5 ng/mL lung

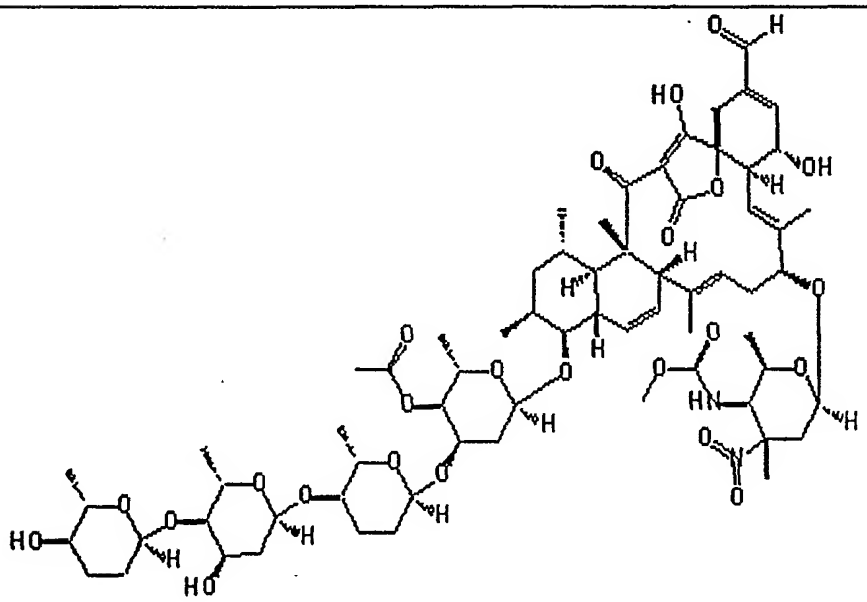


FIG. 11C

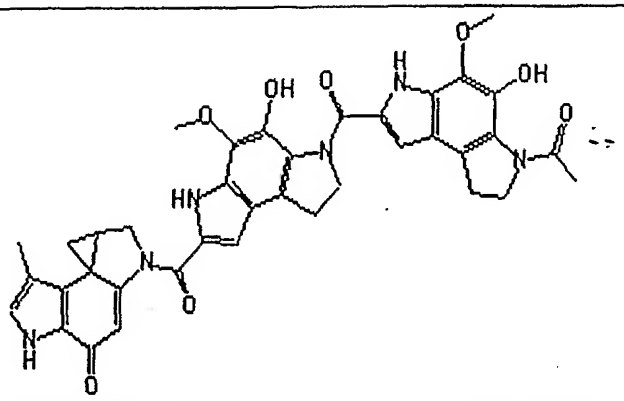
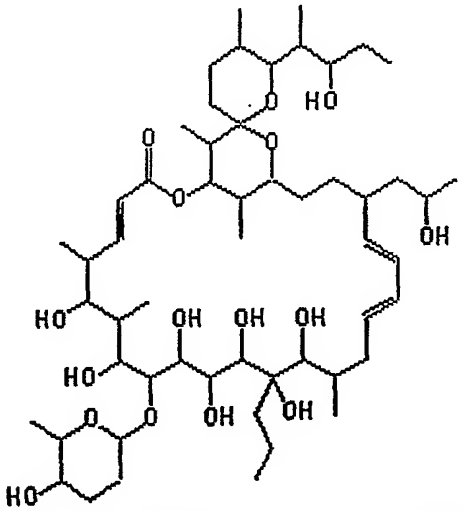
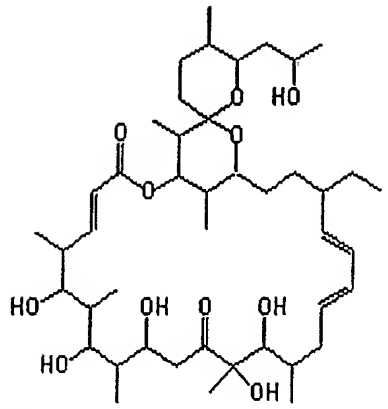
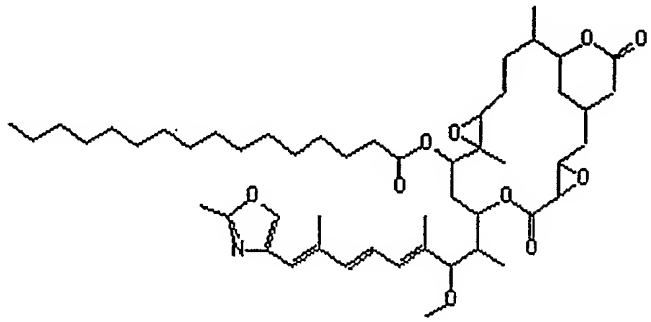
Tetrocarcin A/ not reported/ KF-67544	73666-84-9/ analogs are reported	cancer/ not reported	**inhibits the** anti-apoptotic functino of Bcl2	**not reported**
				
Gilvusmycin/ *Streptomyces* QM16	195052-09-6	cancer/ not reported	not reported	IC50's in ng/mL: 0.08 P388 0.86 K562 (CML) 0.72 A431 (EC) 0.75 MKN28 (GI); (for all < 1 nM)
				
IB-96212/ marine actinomycete/ IB-96212	220858-11-7/ IB-96212; IB-98214; IB-97227	Cancer and Antibacterial/ not reported	not reported	IC50's in ng/mL: 0.1 P388

FIG. 11D



BE-56384[3]/ *Streptomyces* Sp./ BE-56384	207570-04-5	cancer/ not reported	not reported	IC50's in ng/mL: 0.1 P388 0.29 colon 26 34 DLD-1 0.12 PC-13 0.12 MKM-45



Palmitoylrhizoxin/ semi-synthetic; [4]*Rhizopus chinensis*	**135819-69-1/** Analog of rhizoxin	**cancer/** binds LDL; less cytotoxic than rhizoxin	**tubulin binding agent (cell cycle inhibitor)**	**not reported**

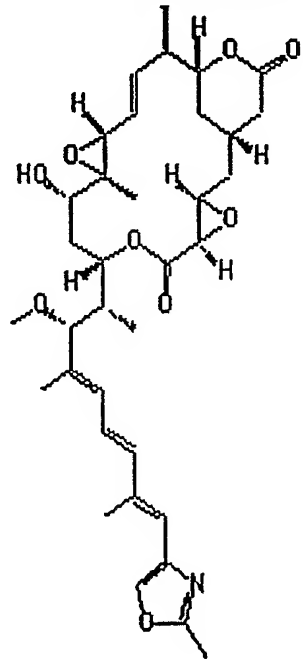


FIG. 11E

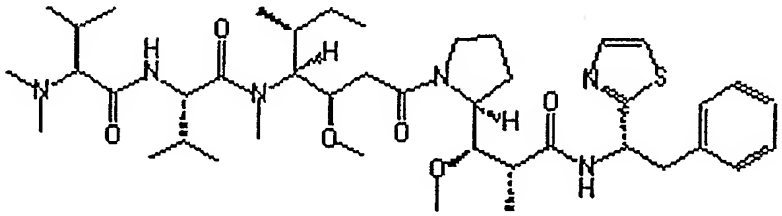
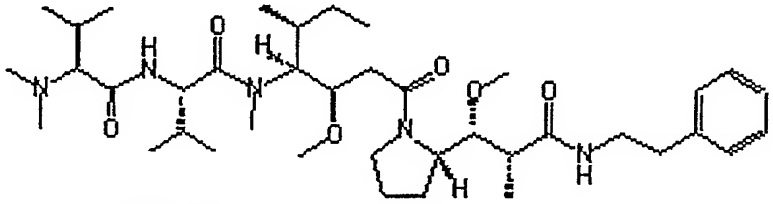
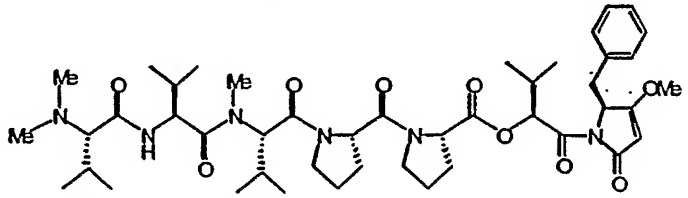
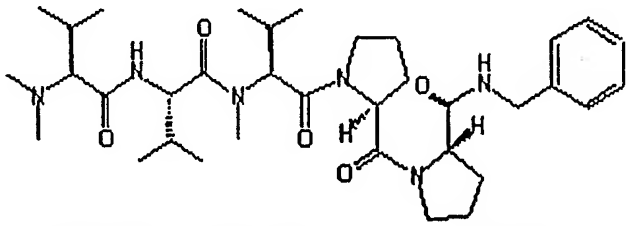
Rhizoxin/ *Rhizopus chinensis*/ WF-1360; NSC-332598; FR-900216	95917-95-6; 90996-54-6	melanoma, lung, CNS, colon, ovary, renal, breast, head and neck/ Rapid Drug clearance; High AUC correlates with high toxicity	tubulin binding agent (cell cycle inhibitor)	NCI tumor panel (NSC 332598); log GI50's: 50 nM to 50 fM; log LC50's: 50 μM to 0.5 nM (several cell lines at 50 fM).
				
Dolastatin-10/ *Dolabella auricularia* (sea hare)/ NSC-376128	110417-88-4/ other Dolistatins (ie. 15) and analogs	prostate, melanoma, leukemia/ myelotoxicity (at greater than 0.3 pM)	tubulin binding (tubulin aggregation)	NCI tumor panel (60 cell line; GI50); 25 nM to 1 pM (most < 1 nM) (three cell lines μM)
				
soblidotin/ synthetic/ TZT-1027; auristatin PE	149606-27-9/ analogs prepared	cancer (pancreas, esophageal colon, breast, lung, etc) / MTD was 1.8 mg/Kg (IV); toxicity not reported	tubulin binding agent	cell culture: colon, melanoma, M5076 tumors, P388 with 75-85% inhibition (dose not reported)
				
Dolastatin-15/ *Dolabella auricularia* (sea hare)	not reported/ other Dolistatins (ie. 15) and analogs	cancer/ not reported	Tubulin binding (tubuline aggregation)	NCI tumor panel (60 cell line; GI50); 25 nM to 39 pM (most < 1 nM) (one cell line 2.5 μM); most active in breast
				
Cemadotin[4]/	1159776-69-	melanoma/	tubulin	NCI tumor panel (NCS

FIG. 11F

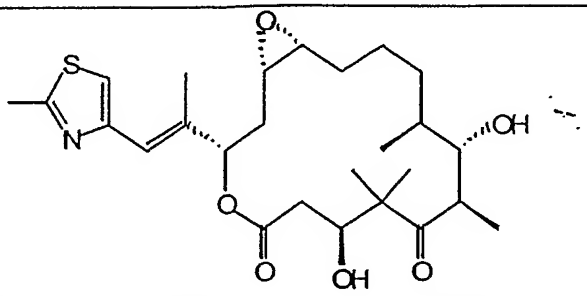
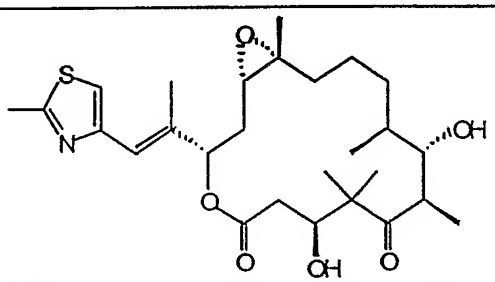
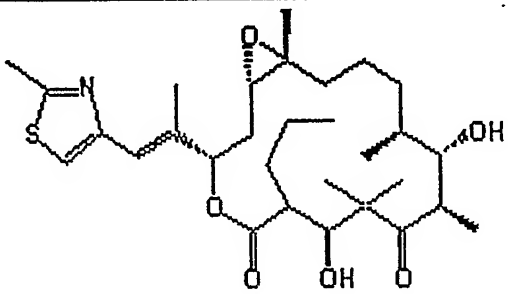
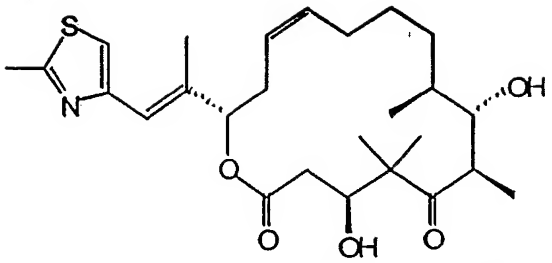
Synthetic; Parent Dolastatin-15 was isolated from *Dolabella auricularia* (sea hare)/ LU-103793; NSC D-669356	9/ many analogs	**hypertension, myocardial** ischemia and myelosuppression were dose-limiting toxicities.	**binding** (tubulin aggregation)	**D-669356); active in** breast, ovary, endometrial, sarcomas and drug resistant cell lines. Data not public.
				
Epothilone A/ Synthetic or isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)	not reported/ many analogs	cancer/ not reported	tubulin binding (tubulin polymeriza-tion)	IC50's of; 1.5 nM MCF-7 (breast) 27.1 nM MCF-7/ADR 2.1 nM KB-31 (melanoma) 3.2 nM HCT-116
				
Epothilone B/ Synthetic or isolated from *Sorangium cellulosum* (myxococcales) strain So ce90) / EPO-906	152044054-7/ many analogs	Solid tumors (breast, ovarian, etc)/ well tolerated; t1/2 of 2.5 hrs; partial responses (phase I); diarrhea major side effect.	tubulin binding (tubulin polymeriza-tion)	IC50's of; 0.18 nM MCF-7 (breast) 2.92 nM MCF-7/ADR 0.19 nM KB-31 (melanoma) 0.42 nM HCT-116; broad activity reported
				
Epothilone Analog / Synthetic or semi-synthetic; Original lead, Epothilone A, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ ZK-EPO	not reported / hundreds of analogs	cancer/ not reported	tubulin binding (tubulin polymeriza-tion)	IC50's of 0.30 to 1.80 nM in various tumor cell lines; active in drug resistant cell lines

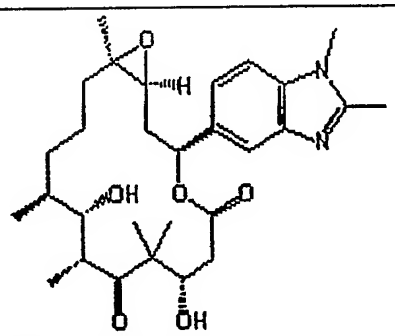
FIG. 11G



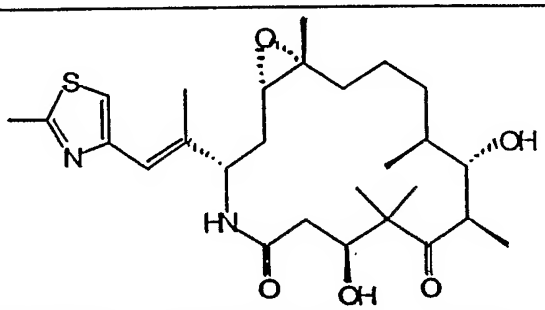
Epothilone D / Epothilone D, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ KOS-862	189452-10-9/ many analogs	Solid tumors (breast, ovarian, etc)/ emesis and anemia; t1/2 of 5-10 hrs.	tubulin binding (tubulin polymeriza-tion)	NCI tumor panel (NSC-703147; IC50); 0.19 nM KB-31 (melanoma) 0.42 nM HCT-116; broad activity reported

Structure Not Identified

Epothilone D analog [5]/ Synthetic or semi-synthetic; Original lead, Epothilone D, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ KOS-166-24	189453-10-9/ hundreds of analogs	Solid tumors; not reported	tubulin binding (tubulin polymeriza-tion)	not reported



Epothilone Analog / Synthetic; Original lead, Epothilone A, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ CGP-85715	not reported/ hundreds of analogs	cancer; not reported	tubulin binding (tubulin polymeriza-tion)	not reported



Epothilone Analog/	219989-84-1/	non-small cell Lung,	tubulin	NCI tumor Panel (NSC-

FIG. 11H

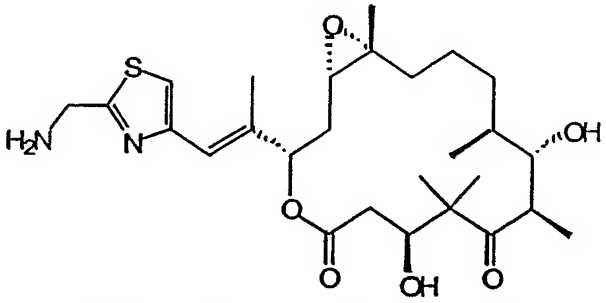
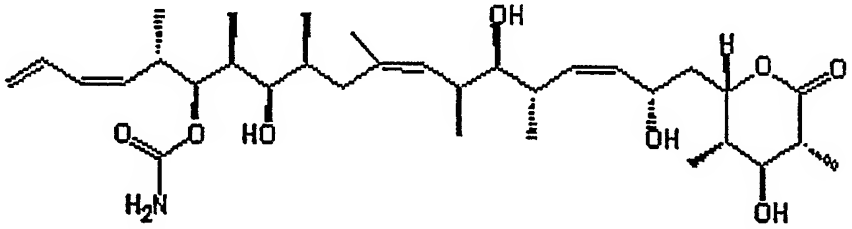
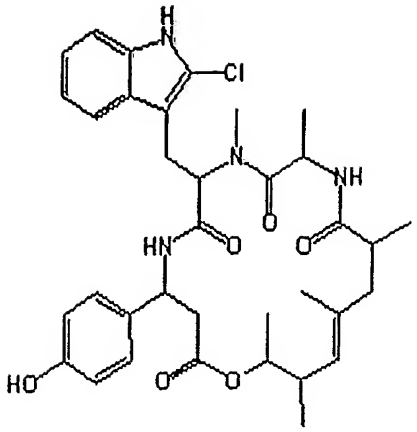
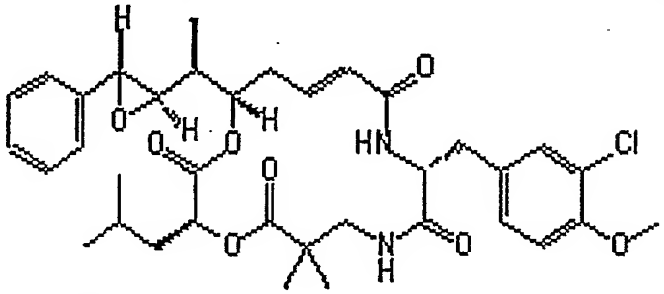
Synthetic or semi-synthetic; Original lead, Epothilone B, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ BMS-247550	hundreds of analogs	breast, stomach tumor (objective responses in breast ovarian and lung)/ sever toxicity (fatigue, anorexia, nauseas, vomiting, neuropathy myalgia)	binding (tubulin polymerizati on)	710428 & NSC-710468); 8-32 nM (NCI data not available)
				
Epothilone Analog / Synthetic or semi-synthetic; Original lead, Epothilone B, isolated from *Sorangium cellulosum* (myxococcales) strain So ce90)/ BMS-310705	not reported/ hundreds of analogs	advanced cancers/ adverse events (diarrhea, nausea, vomiting , fatigue, neutropenia); t1/2 of 3.5 hrs; improved water solubility to BMS 247550.	tubulin binding (tubulin polymeriza-tion)	broad activity with IC50's of 0.7 to 10 nM
				
Discodermolide / synthetic; orginally isolated from *Discodermia dissoluta* (deep water sponge); rare compound (7 mg per 0.5 Kg sponge/ XAA-296	127943-53-7/ analogs less potent	solid tumors/ not reported; 100-fold increase in water solubility over taxol	tubulin stabilizing agent (similar to taxol)	Broad activity (A549-nsclung, prostate, P388, ovarian with IC50's about 10 nM) including multi-drug resistant cell lines;

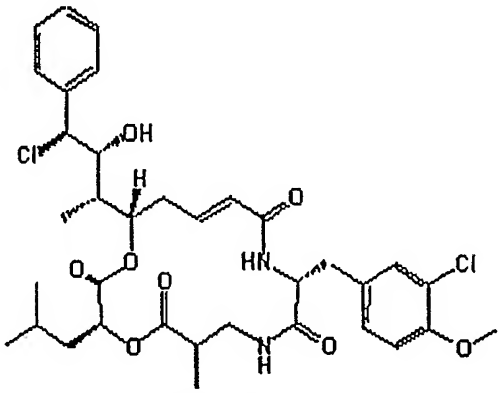
FIG. 11I



Chondramide D/ not reported	172430-63-6	cancer/ not reported	tubulin binding agent; actin polymeriza-tion inhibitor	5 nM A-549 (epidermoid carcinoma) 15 nM A-498 (kidney) 14 nM A549 (lung) 5 nM SK-OV-3 (ovary) 3 nM U-937 (lymphoma)



Cryptophycin analogs (including 52, 55 and others)[6]/ *Nostoc* sp GSV 224 (blue-green algae) isolated Cryptophycin 1./ LY-355703; Ly-355702; NSC-667642	204990-60-3 and 186256-67-7/ many potent analogs prepared at Lilly	solid tumors, colon cancer/ Phase II studies halted because of severe toxicity with one death resulting from drug;	tubulin polymeriza-tion inhibitor	broad activity (lung, breast, colon, leukemia) with IC50's of 2 to 40 pM; active against multi-drug resistance cell lines (resistant to MDR pump). NCI tumor panel, GI50's from 100 nM to 10 pM; LC50's from 100 nM to 25 pM.



Cryptophycin 8/	168482-36-8;	solid tumors/	tubulin	broad spectrum

FIG. 11J

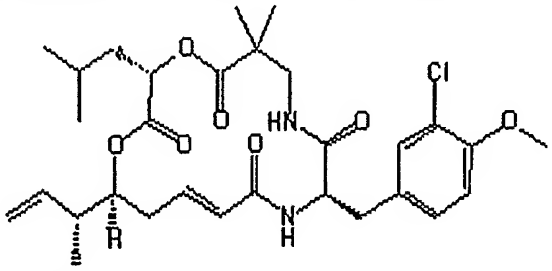
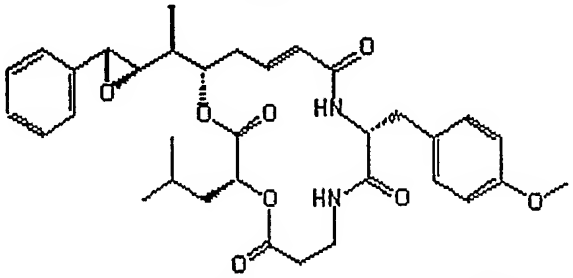
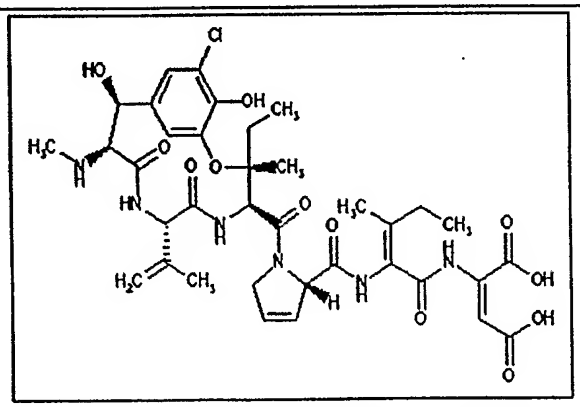
semi-synthetic; starting material from *Nostoc* sp.	168482-40-4; 18665-94-1; 124689-65-2; 125546-14-7/ cryptophycin 5, 15 and 35	not reported	polymerization inhibitor	anticancer activity (cell culture) including multi-drug resistant tumors
Cryptophycin analogs[7]/ synthetic; semi-synthetic, starting material from *Nostoc* sp./ LY-404291	 219660-54-5/ LY-404292	solid tumors/ not reported	topoisomerase inhibitors	not reported
Arenastatin A analogs[8]/ *Dysidea arenaria* (marine sponge)/ Cryptophycin B; NSC-670038	 not reported/ analogs prepared	cancer/ not reported	inhibits tubulin polymerization	8.7 nM (5 pg/mL) KB (nasopharyngeal); NCI tumor panel (GI50's); 100 pM to 3 pM
Phomopsin A/ *Diaporte toxicus* or *Phomopsin leptostromiformis* (fungi)	 not reported	Liver cancer (not as potent in other cancers)/ not reported	tubulin binding agent	potent anticancer activity especially against liver cancer

FIG. 11K

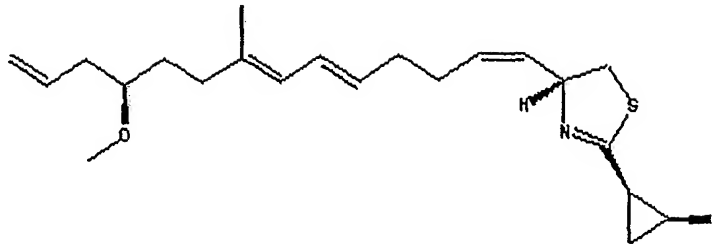
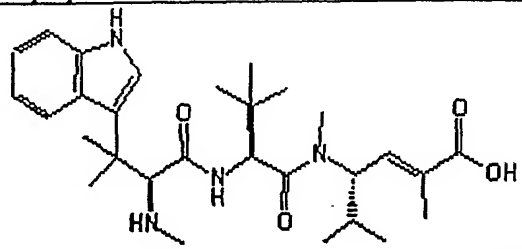
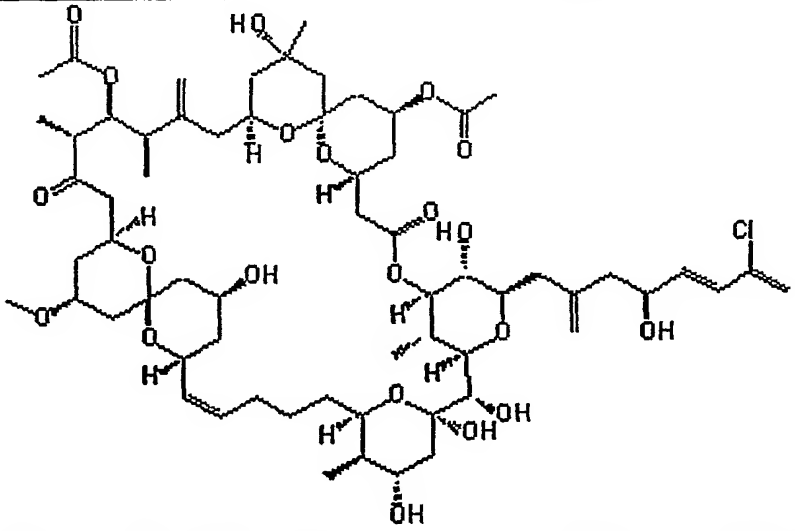
				
Curacin A and analogs/ *Lyngbya majuscula* (blue green cyanobacterium)	155233-30-0/ analogs have been prepared	Cancer/ not reported	Tubulin binding agent	broad activity (cancer cell lines); 1-29 nM
				
Hemiasterlins A & B and analogs[9]/ *Cymbastela* sp.	not reported/ criamide A & B; geodiamiolid-G	Cancer/ not reported	Antimitotic agent (tubulin binding agent)	broad activity: 0.3-3 nM MCF7 (breast); 0.4 ng/mL P388
				
Spongistatins (1-9)[10]/ *Spirastrell spinispirulifera* (sea sponge)	149715-96-8; 158734-18-0; 158681-42-6; 158080-65-0; 150642-07-2; 153698-80-7; 153745-94-9; 150624-44-5; 158734-19-1/ other spongistatins	cancer/ not reported	tubulin binding agent	Most potent compounds ever tested in NCI panel cell line (mean GI50's of 0.1 nM; Spongistatin-1 GI50's of 0.025-0.035 nM with extremely potent activity against a subset of highly chemoresistant tumor types

FIG. 11L

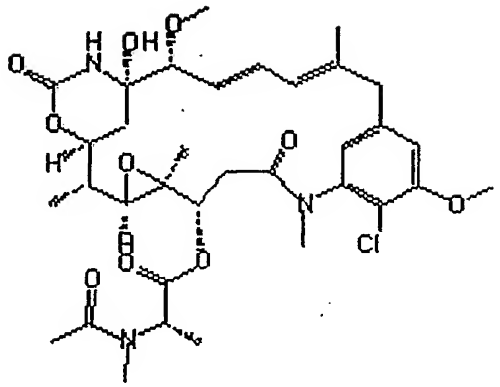
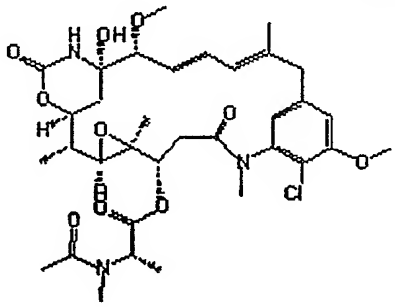
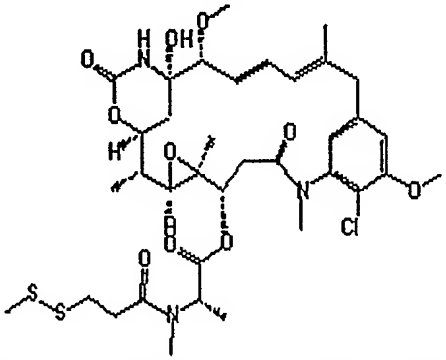
				
Maytansine/ *Maytenus* sp./ NSC-153858	35846-53-8/ other related macrolides	cancer/ severe toxicity	tubulin binding agent (causes extensive disassembly of the microtubule and totally prevents tubulin spiralizaiton)	Broad Activity in NCI tumor panel (NSC-153858; NSC-153858); NCI tumor panel, GI50's from 3 μM to 0.1 pM; LC50's from 250 μM to 10 pM. Two different experiments gave very different potencies.
				
Maytansine-IgG(EGFR directed)-conjugate[11]/ semi-synthetic; starting material from *Maytenus* sp.	not reported/ other related macrolides	breast , head and neck, Squamous cell carcinoma/ not reported	EGFR binding and tubulin binding	not reported
				
Maytansine-IgG(CD56 antigen)-conjugate[12], 3.5 drug molecules per IgG/ semi-synthetic; starting material from *Maytenus*	not reported/ other related macrolides	Neuroendocrine, small-cell lung, carcinoma/ mild toxicity (fatigue, nausea, headaches and mild peripheral	CD56 binding and tubulin binding	antigen-specific cytotoxicity (cell culture; epidermal, breast, renal ovarian colon) with IC50's of

FIG. 11M

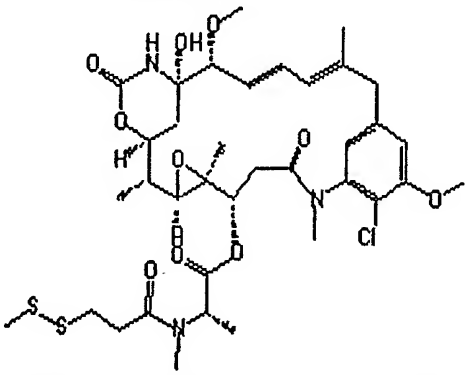
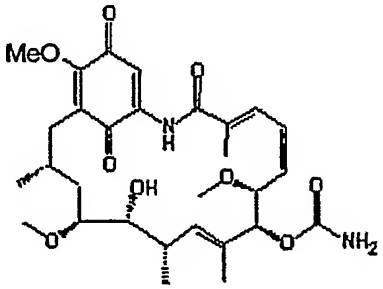
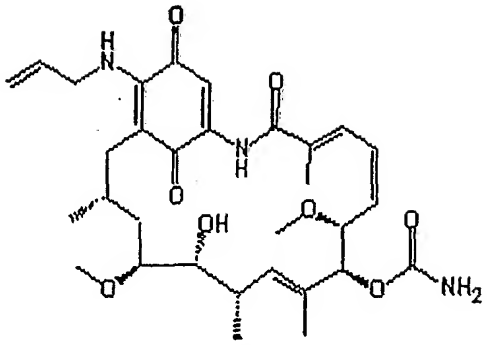
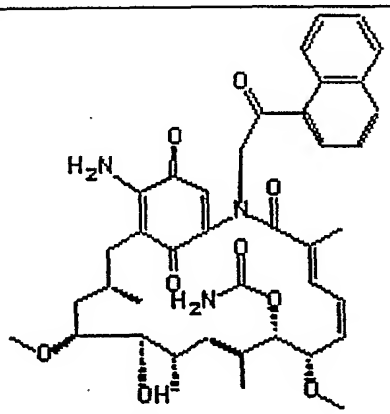
sp./ huN901-DM1		neuropathy); no hematological toxicity; MTD 60 mg/Kg, I.V., weekly for 4 weeks; only stable disease reported (humans)		10-40 pM; animal studies (miceSCLC tumor--alone and in combination with taxol or cisplatin completely eliminated tumors).
				
Maytansine-IgG(CEA antigen)-conjugate[13], 4 drug molecules per IgG/ semi-synthetic; starting material from *Maytenus* sp./ C424-DM1	not reported/ other related macrolides	non-small-cell lung, carcinoma pancreas, lung, colon/ mild toxicity (fatigue, nausea, headaches and mild peripheral neuropathy); pancreatic lipase elevated; MTD 88 mg/Kg, I.V., every 21 days; only stable disease reported (humans); t1/2 was 44 hr.	CEA binding and tubulin binding	antigen-specific cytotoxicity (cell culture; epidermal, breast, renal ovarian colon) with IC50's of 10-40 pM; animal studies (mice: melanoma [COLO-205]--alone and in combination with taxol or cisplatin completely eliminated tumors);
				
Geldanamycin / *Streptomyces hygroscopicus* var. Geldanus/ NSC-212518; Antibiotic U 29135; NSC-122750	30562-34-6/ natural derivatives	cancer/ not reported	binds Hsp 90 chaperone and inhibits function	NCI tumor panel (cell culture); 5.3 to 100 nM; most active in colon, lung and leukemia. NCI tumor panel, GI50's from 10 μM to 0.1 nM; LC50's from 100 μM to 100 nM. Two assays with very different potencies.

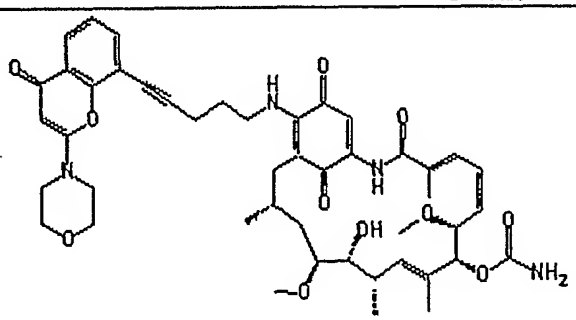
FIG. 11N



Geldanamycin Analog/ semi-synthetic; / CP-127374; 17-AAG; NSC-330507	745747-14-7/ Kosan, NCI and UK looking for analogs with longer t1/2 and oral activity; analogs include: NSC-255110; 682300; 683661; 683663.	solid tumors/ Dose limiting toxicities (anemia, anorexia, diarrhea, nausea and vomiting); t1/2 (i.v.) is about 90 min; no objective responses measured at 88 mg/Kg (i.v. daily for 5 days, every 21 days);	binds Hsp 90 chaperone and inhibits function	cell culture (not reported); animal models active (tumor regression observed) in breast, ovary, melanoma, colon.



Geldanamycin analog/ semi-synthetic; / CP-202567	not reported/ analogs prepared	solid tumors/ not reported	binds Hsp 90 chaperone and inhibits function	not reported



Geldanamycin conjugates/ semi-synthetic; / LY-294002-GM; PI3K-1-GM	345232-44-2/ analogs prepared	breast/ not reported	binds Hsp 90 chaperone and inhibits function; binds and	cell culture (no reported); animal models performed

FIG. 11O

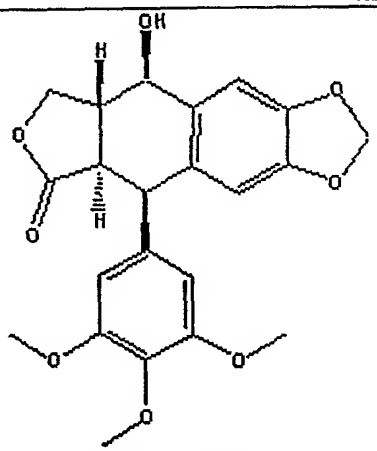
			inhibits PI-3 kinase	
Structure Not Reported				
Geldanamycin Analog/ not reported/ CNF-101	not reported/ analogs prepared	breast, prostate/ not reported	binds Hsp 90 chaperone and inhibits function	not reported
Structure Not Reported				
Geldanamycin-testosterone conjugate/ semi-synthetic/ GMT-1	not reported/ analogs prepared	prostate/ not reported	binds Hsp 90 chaperone and inhibits function and testosterone receptors where it is internalized	not reported; conjugate has a 15-fold selective cytotoxicity for androgen positive prostate cells
				
Podophyllotoxin/ *Podophyllum* sp.	518-28-5/ many analogs	Verruca vulgaris, Condyloma/ severe toxicity when given i.v. or s.c.	tubulin inhibitor and topoisomer-ase inhibitor	broad activity (cell culture) with IC50's in μM range

FIG. 11P

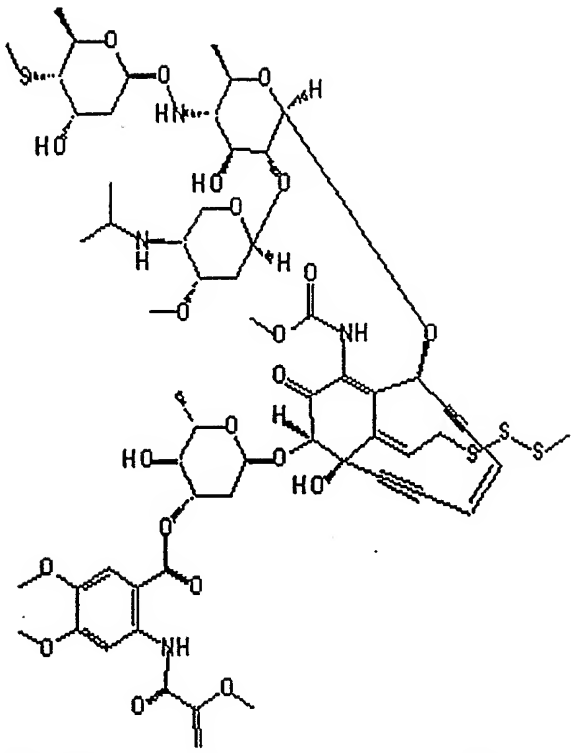
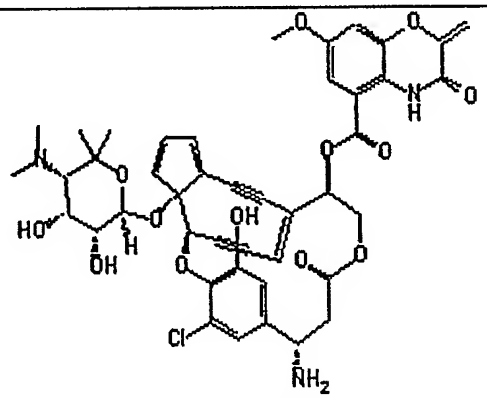
				
esperamicin-A1/ not known/ BBM-1675A1; BMY-28175; GGM-1675	99674-26-7	cancer/ not reported (suspected severe toxicity)	DNA cleaving agent	highly potent activity (cell culture); animal models highly potent with optimal dose of 0.16 micrograms/Kg
				
C-1027[14]/ *Streptomyces setonii* C-1027/ C-1027	120177-69-7	cancer (examined hepatoma, breast, lung and leukemia/ not reported	DNA cleaving agent	extremely potent (cell culture) IC50's in pM and fM; conjugated to antibodies the potency remains the same (ie. 5.5 to 42 pM);

FIG. 11Q

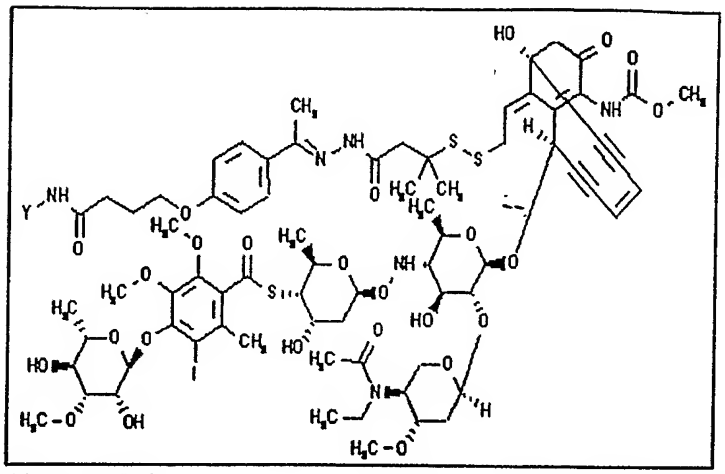
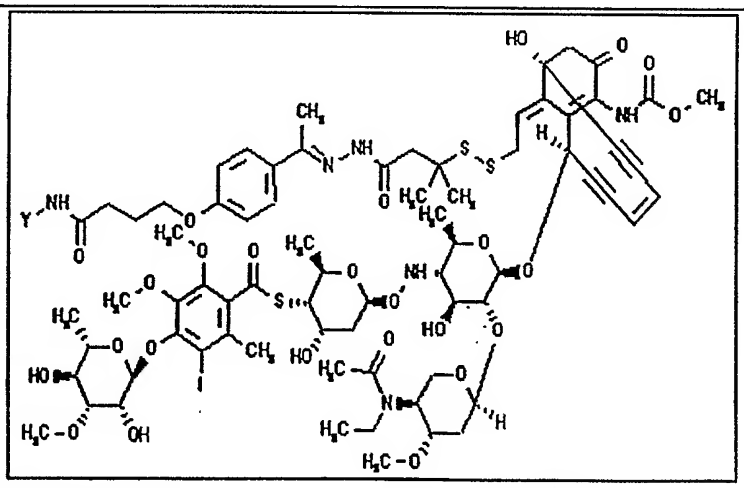
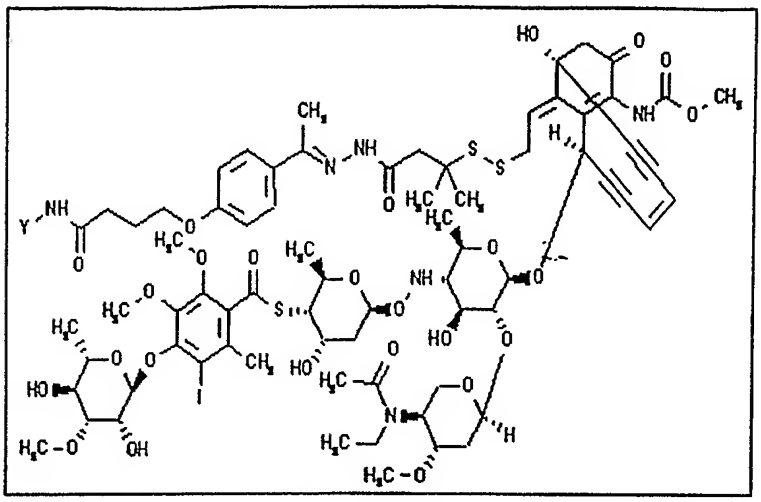
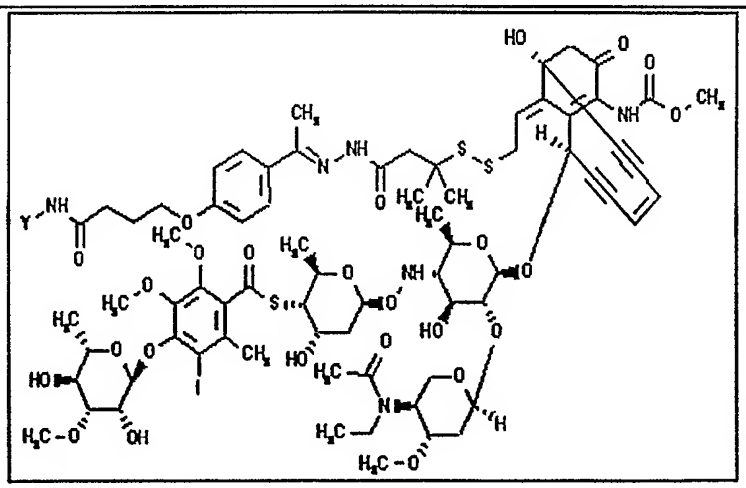
$Pr(X\text{-}S\text{-}S\text{-}W)_m$ m = 0.5 - 15 Pr = proteinaceous carrier W = calicheamicin minus Me-S-S-S X = linker Y = antibody P76.6				
Calicheamicin-IgG(CD33 antigen)-conjugate[15]/ semi-synthetic: *Micromonospora echinospora*/ gemtuzumab ozogamicin; mylotarg; WAY-CMA-676; CMA-676; CDP-771	113440-58-7; 220578-59-6/ several reported in patents	AML/ mild toxicity	DNA cleaving agent	Kills CD33+ cells (HL-60, NOMO-1, and NKM-1) at 100 ng/mL; MDR cell lines are not effected by the drug.
$Pr(X\text{-}S\text{-}S\text{-}W)_m$ m = 0.5 - 15 Pr = proteinaceous carrier W = calicheamicin minus Me-S-S-S X = linker Y = antibody P76.6				
Calicheamicin-IgG-conjugates[16]/ semi-synthetic: *Micromonospora echinospora*	113440-58-7; 220578-59-6	cancer/ not reported	DNA cleaving agent	TBD

FIG. 11R

Pr(-X-S-S-W)$_m$ m = 0.5 - 15 Pr = proteinaceous carrier W = calicheamicin minus Me-S-S-S X = linker Y = antibody P76.6 				
Calicheamicin-IgG(OBA1 antigen) conjugate/ semi-synthetic: *Micromonospora echinospora*/ OBA1-H8	not reported	cancer/ not reported	DNA cleaving agent	all human cancer; data not reported
Pr(-X-S-S-W)$_m$ m = 0.5 - 15 Pr = proteinaceous carrier W = calicheamicin minus Me-S-S-S X = linker Y = antibody P76.6 				
Calicheamicin-IgG(CD22 antigen) conjugate/ semi-synthetic: *Micromonospora echinospora*/ CMC-544	not reported	non-Hodgkin lymphoma, cancer/ not reported	DNA cleaving agent	all human cancer; data not reported

parially esterified polystyrene maleic acid copolymer (SMA) conjugated to neocarzinostatin (NCS)

Neocarzinostatin[17]/ semi-synthetic; *Streptomyces carconistaticus*/ Zinostatin stimalamer; YM-881; YM-16881	123760-07-6; 9014-02-2	liver cancer and brain cancer/ not reported	DNA cleaving agent	cell culture data not reported.

FIG. 11S

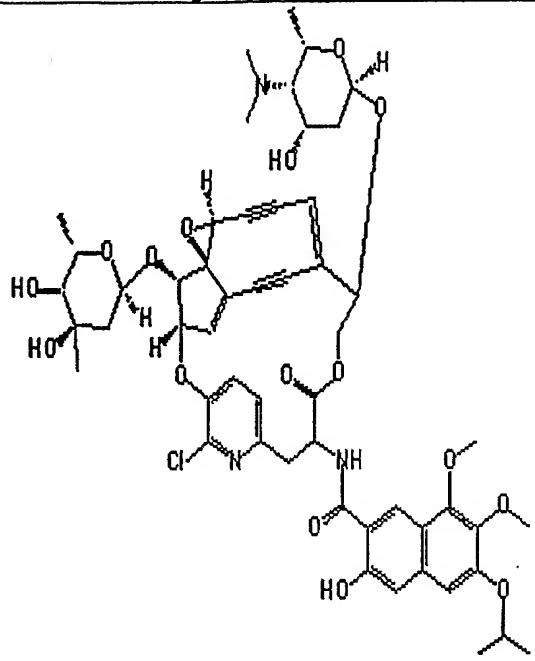
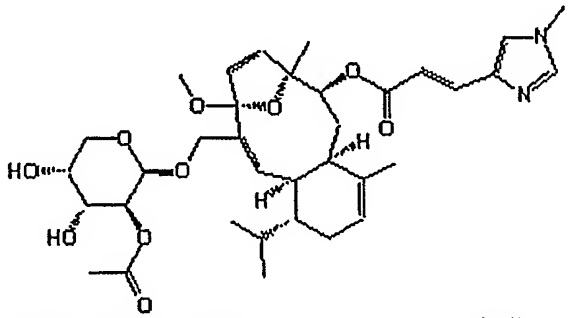
		IgG (TES-23)-conjugated to neocarzinostatin		
Neocarzinostatin/ not reported/ TES-23-NCS	not reported	solid tumors/ toxicity not reported; the TES-23 antibody (without anticancer agent) was as effective at eliminating tumors as the drug conjugated protein	DNA cleaving agent and immunostim-ulator	cell culture data not reported.
				
Kedarcidin[18]/ *Streptoalloteichus* sp NOV strain L5856, ATCC 53650/ NSC-646276	128512-40-3; 128512-39-0/ chromophore and protein conjugate	cancer/ not reported	DNA cleaving agent	cell culture (IC50's in ng/mL), 0.4 HCT116; 0.3 HCT116/VP35; 0.3 HCT116/VM46; 0.2 A2780; 1.3 A2780/DDP. animal models in P388 and B-16 melanoma. NCI tumor panel, GI50's from 50 μM to 5 μM.
				
Eleutherobins/ marine coral	174545-76-7/ sarcodictyins (marine coral)	cancer/ not reported	tubulin binding agent	similar potency to taxol; not effective against MDR cell lines

FIG. 11T

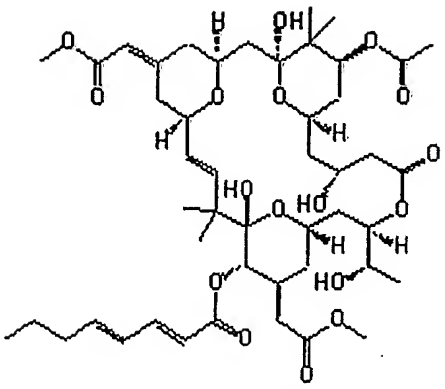
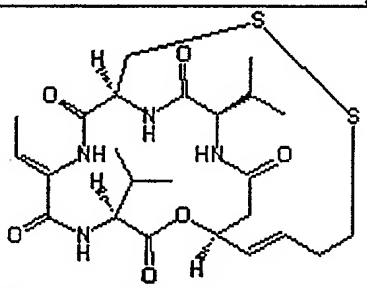
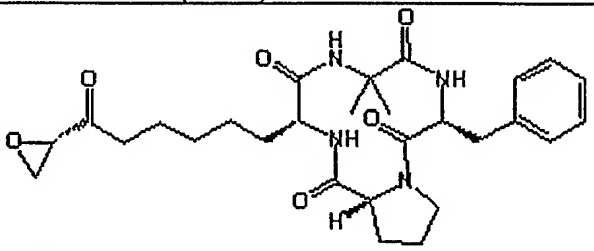
				
Bryostatin-1/ *Bugula neritina* (marine bryosoan)/ GMY-45618; NSC-339555	83314-01-6	leukemia, melanoma, lung, cancer/ myalgia; accumulated toxicity; poor water solubility; dose limiting toxicity	immunostim-ulant (TNF, GMCSF, etc); enhances cell kill by current anticancer agents	not reported
				
FR-901228/ *Chromobacterium violaceum* strain 968/ NSC-63-176; FK-228	128517-07-7	leukemia, T-cell lymphoma, cancer/ toxic doses (LD50) 6.4 and 10 mg/Kg, ip and iv respectively; GI toxicity, lymphoid atrophy; dose limiting toxicity (human) 18 mg/Kg; t1/2 of 8 hrs (human)	histone deacetylase inhiibitor	In vitro cell lines (NCI tumor panel); IC50's of between 0.56 and 4.1 nM (breast, lung, gastric colon, leukemia)
				
Chlamydocin/ not reported	53342-16-8	cancer/ not reported	histone deacetylase inhiibitor	not reported (cell culture); inhibits histone deacetylase at an IC50 of 1.3 nM

FIG. 11U

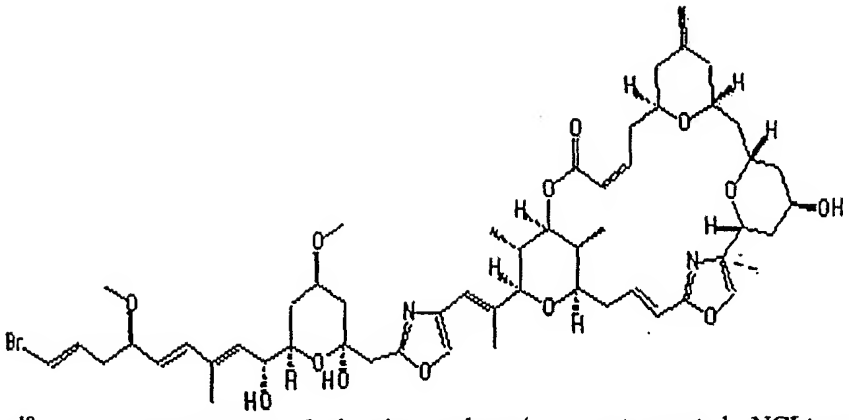
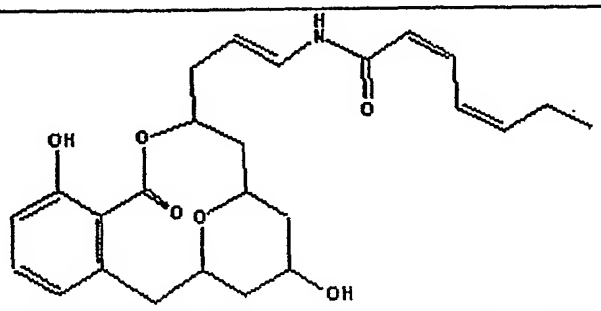
 **Phorboxazole A**[19]/ marine sponge	181377-57-1; 165689-31-6; 180911-82-4; 165883-76-1/ analogs prepared	leukemia, myeloma/ not reported	not reported (induces apoptosis)	NCI tumor panel (details not reported); IC50's of 1-10 nM. The inhibition values (clonogenic growth of human cancer cells) at 10 nM ranged from 6.2 to > 99.9% against NALM-6 human B-lineage acute lymophoblastic leukemia cells, BT-20 breast cancer cells and U373 glioblastoma cells, with the specified compound showing inhibition values in the range of 42.4 to > 99.9% against these cell lines.; IC50's are nM for MDR cell lines.
 **Apicularen A**/ *Chondromyces robustus*	220757-06-2/ natural derivatives	cancer/ not reported	not reported	IC50's of 0.1 to 3 ng/mL (KB-3-A, KB-Va, K562, HL60, U937, A498, A549, PV3 and SK-OV3)

FIG. 11V

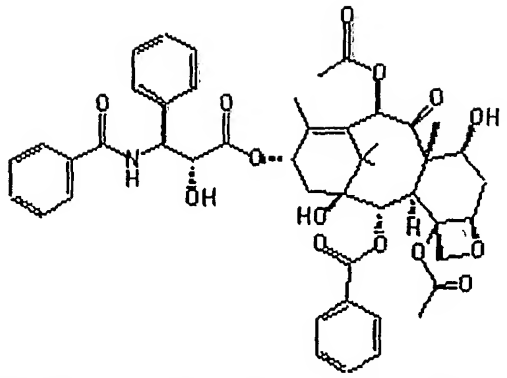
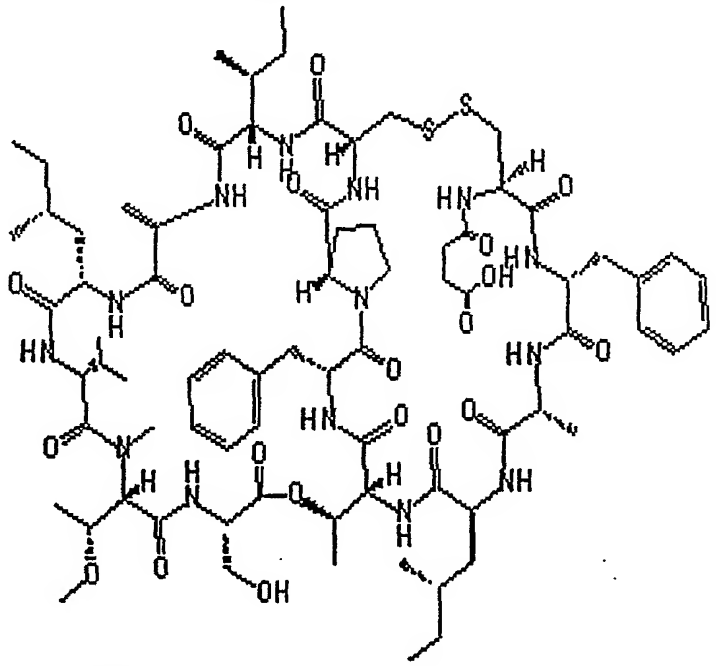
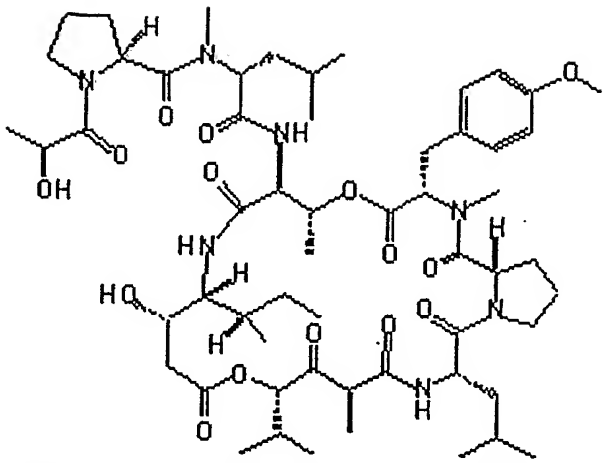
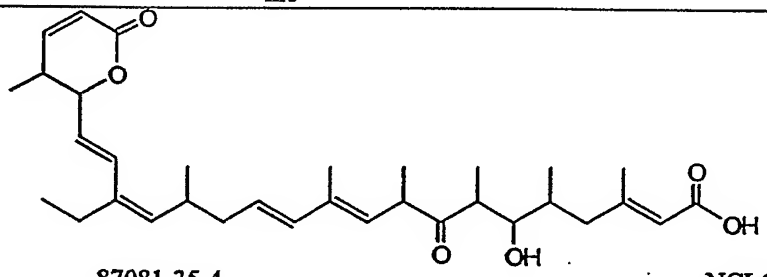
				
Taxol/ Pacific yew and fungi/ Paclitaxel; NSC-125973	33069624/ many analogs	cancer; breast, prostate, ovary, colon, lung, head & neck, etc./ severe toxicity (grade III and IV)	tubulin binding agent	NCI tumor panel; GI50's of 3 nM to 1 μM; TGI 50 nM to 25 μM
				
Vitilevuamide/ *Didemnum cuculliferum* or *Polysyncraton lithostrotum*	191681-63-7	cancer/ not reported	tubulin binding agent	cell culture; IC50's of 6-311 nM (panel of tumor cell lines HCT116 cells, A549 cells, SK-MEL-5 cells A498 cells). The increase in lifespan (ILS) for CDF1 mice after ip injection of P388 tumor cells was in the range of -45 to +70% over the dose range of 0.13 to 0.006 mg/kg.

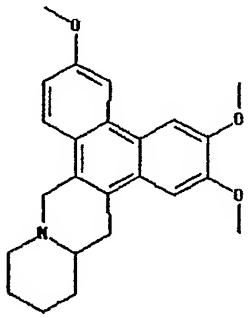
FIG. 11W



Didemnin B/ *Tridemnum solidum*/ NSC-2325319; IND 24505	77327-05-0; 77327-04-9; 77327-06-1/ other related natural products	non-Hodgkin's lymphoma, breast, carcinoma, CNS, colon/ Discontinued due to cardiotoxicity; nausea, neuro-muscular toxicity and vomiting MTD 6.3 mg/Kg; toxicity prevented achieving a clinically signif. effect; rapidly cleared (t1/2 4.8 hrs	inhibits protein synthesis via EF-1	NCI 60-tumor panel (GI50's): 100 nM to 50 fM. Not potent against MDR cell lines.



Leptomycin B/ *Streptomyces* sp. strain ATS 1287/ NSC-364372; elactocin	87081-35-4	NCI 60-tumor panel (GI50's): 8 μM to 1 pM; (LC50): 250 μM to 10 nM (several cell lines at 0.1 nM). Two testing results with very different potencies.



Cryptopleurin/	NCI 60-tumor panel

FIG. 11X

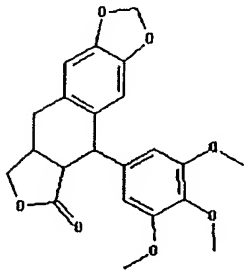
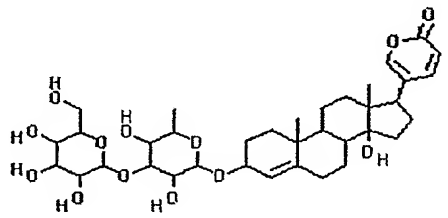
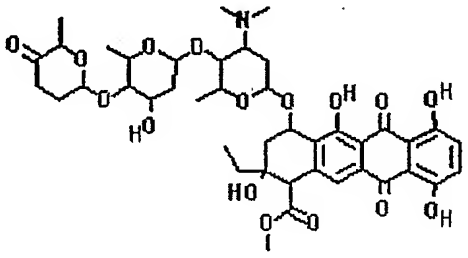
not known/ NSC-19912		(GI50's): 19 nM to 1 pM; (LC50): 40 μM to 10 nM (several cell lines at 1 pM).
		
Silicicolin/ not known/ NSC-403148, deoxypodophyllotoxin, desoxypodophyllotoxin podophyllotoxin, deoxysilicicolin	19186-35-7	NCI 60-tumor panel (GI50's): ~100 nM to 3 nM; (LC50): 50 μM to 10 nM
		
Scillaren A/ not known/ NSC-7525; Gluco-proscillaridin A; Scillaren A	124-99-2	NCI 60-tumor panel (GI50's): 50 nM to 0.1 nM; (LC50): 250 μM to 0.1 nM
		
Cinerubin A-HCl/ not known/ NSC-243022; Cinerubin A hydrochloride; CL 86-F2 HCl; CL-86-F2-hydrochloride	not reported	NCI 60-tumor panel (GI50's): 15 nM to 10 pM; (LC50): 100 μM to 6 nM

FIG. 11Y